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13. ABSTRACT (Maximum 200 Words)
Metastasis is the major cause of death in breast cancer patients and is partly caused by the action of proteolytic enzymes that degrade extracellular matrix (ECM). We have focused on the gelatinases, MMP-2, and MMP-9, two ECM-degrading enzymes that are members of the matrix metalloproteinase (MMP) family of proteases. The gelatinases are associated with the surface of breast cancer cells. MMP-2 surface binding plays a role in activation by MT1-MMP, a membrane-bound MMP that is also expressed in breast cancer cells. MMP-2 activation is mediated by the action of TIMP-2, a metalloproteinase inhibitor. We characterized in detail the process of pro-MMP-2 activation by MT1-MMP and demonstrated the role of TIMP-2 in this process. The regulation of MT1-MMP on the cell surface was also investigated. These studies demonstrated that MT1-MMP undergoes autocatalytic processing and ectodomain shedding, which serve to control the level of MT1-MMP on the cell surface and produce active enzyme at both the cell membrane and in the extracellular space. MT1-MMP initiates a cascade of zymogen activation on the cell surface that leads to the generation of active MMP-2 and active MMP-9. This process is regulated by TIMP-2. A novel mechanism-based inhibitor for the gelatinases that binds with high affinity and irreversible has been characterized. Together, these studies have defined some key aspects that regulate the function of the MT1-MMP/gelatinase axis on the surface of breast cancer cells and develop new approaches to counteract their action in tumor tissues.

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INTRODUCTION

Breast cancer metastasis depends among other factors on the expression and activity of proteolytic enzymes, which helps tumor cells to invade neighboring tissues and eventually form distant metastasis (*1, 2*). A key family of proteases that has been shown to play a critical a role in breast cancer metastasis is the matrix metalloproteinases (MMPs) (*3*). Because of their importance in cancer metastasis, there is considerable interest in developing new approaches for inhibiting their activity in tumor tissues (*4*). Therefore, understanding MMP function and inhibition may yield new ideas for targeting these important enzymes in breast cancer patients. The MMPs cleave all components of the extracellular matrix (ECM) and also non-ECM proteins, which are mostly localized on the cell surface (*5*). Therefore, a key aspect of proteolytic degradation in cancer cells involves the targeting of enzymes to the cell surface (*6, 7*). To achieve their full potential in pericellular proteolysis, the members of the MMP family evolved into secreted and membrane-tethered multidomain enzymes by incorporating distinct domains that facilitate binding to ECM components and surface molecules in the case of soluble MMPs such as the gelatinases (MMP-2 and MMP-9) and unique domains that anchors the enzyme to the cell surface, in the case of the MT-MMPs (*8*). The purpose and scope of the Academic Award proposal was to understand the cell surface regulation of the gelatinases, MMP-2 and MMP-9, and the membrane-anchored MT1-MMP with the goal to decipher the function and role of these enzymes in breast cancer progression.

BODY

Summary of accomplishment of Year 1: 1999-2000

- Characterized the role of TIMP-2 in pro-MMP-2 activation by MT1-MMP
- Completed the characterization of the dimeric and monomeric forms of pro-MMP-9
- Investigated the role of surface binding of pro-MMP-9 in activation of zymogen
- Initiated search of MMP-9 binding proteins using a phage display system

Summary of accomplishment of Year 2-3: 2000-2002

- Characterized the processing of MT1-MMP and described the major enzyme forms

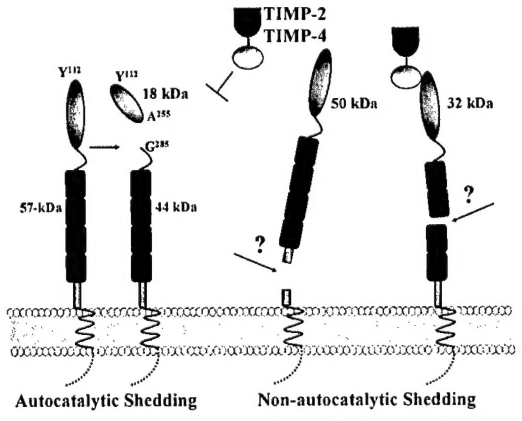
- Reported the synergistic and enhancing effects of TIMP-2 and synthetic MMPIs in pro-MMP-2 activation
- Showed that TIMP-4 cannot promote pro-MMP-2 activation by MT1-MMP
- Demonstrated the localization of MMP-2 and MT1-MMP in caveolae
- Carried out a search for MMP-9 binding proteins using a phage display system

Detail Report of Research for Year 2002-2003

1. <u>Studies on MT1-MMP and MMP-2 Regulation.</u> (J. Biol. Chem., 277: 26340-26350, 2002; J. Biol. Chem., 277: 11201-11207, 2002; Biochem. J., 374: 739-745, 2003, J. Amer. Chem Soc. 22: 6799-6800, 2000).

1A. During the last two years of the granting period we focus in understanding the function of MT1-MMP on the cell surface. MT1-MMP is a type I transmembrane MMP shown to play a critical role in normal development and in malignant processes, including breast cancer (*9*). Emerging evidence indicates that MT1-MMP is regulated by a process of ectodomain shedding. Active MT1-MMP undergoes autocatalytic processing on the cell surface leading to the formation of an inactive 44-kDa fragment and release of the entire catalytic domain. Analysis of the released MT1-MMP forms in various cell types revealed a complex pattern of shedding involving two major fragments of 50 and 18 kDa and two minor species of 56, and 31-35 kDa. Protease inhibitor studies and a catalytically inactive MT1-MMP mutant revealed both autocatalytic (18 kDa) and non-autocatalytic (56, 50, 31-35 kDa) shedding mechanisms. Purification and sequencing of the 18-kDa fragment indicated that it extends from Y^{112} to A^{255}. Structural and sequencing data indicate that shedding of the 18-kDa fragment is initiated at the G^{284}-G^{285} site followed by cleavage between the conserved A^{255} and I^{256} residues near the conserved methionine turn, a structural feature of the catalytic domain of all MMPs. Consistently, a recombinant 18-kDa fragment had no catalytic activity and did not bind TIMP-2. Thus, autocatalytic shedding

<u>Figure 1</u>. Processing of MT1-MMP



evolved as a specific mechanism to terminate MT1-MMP activity on the cell surface by disrupting enzyme integrity at a vital structural site. In contrast, functional data suggest that the non-autocatalytic shedding generates soluble active MT1-MMP species capable of binding TIMP-2. These studies suggest that ectodomain shedding regulates the pericellular and extracellular activities of MT1-MMP through a delicate balance of active and inactive enzyme soluble fragments. Figure 1 describes the processing of MT1-MMP with the major cleavages sites that result in autocatalytic processing. We are currently investigating the precise nature of the species generated by the non-autocatalytic processing. Using a novel approach to isolate active MMPs, using an immobilized synthetic MMP inhibitors we have purified the shed forms of MT1-MMP and send them for sequence analyses. These studies are ongoing.

1B. An important focus of our research was to understand how MT1-MMP activates pro-MMP-2 on the cell surface. If pro-MMP-2 is activated via a ternary complex involving binding of the zymogen to the MT1-MMP/TIMP-2 complex (*10*), how is then the active MMP-2 released from the complex? It is well known that MMP-2 plays a crucial role in extracellular matrix (ECM) degradation associated with cancer metastasis and angiogenesis in breast cancer. The latent form, pro-MMP-2, is activated on the cell surface by the membrane-tethered MMP, membrane type 1-MMP (MT1-MMP) in a process regulated by the tissue inhibitor of metalloproteinase (TIMP)-2. A complex of active MT1-MMP and TIMP-2 binds pro-MMP-2 forming a ternary complex, which permits pro-MMP-2 activation by a TIMP-2-free neighboring MT1-MMP. It remains unclear how MMP-2 activity on the pericellular space is regulated in the presence of TIMP-2. To address this question, the effect of TIMP-2 on MMP-2 activity in the extracellular space was investigated in live cells, and their isolated plasma membrane fractions, engineered to control the relative levels of MT1-MMP and TIMP-2 expression. We show that both free and inhibited MMP-2 is detected in the media, and that the net MMP-2 activity correlates with the level of TIMP-2 expression. Studies to displace MT1-MMP-bound TIMP-2 in a purified system with active MMP-2, show minimal displacement of inhibitor, under the experimental conditions, due to the high affinity interaction between TIMP-2 and MT1-MMP. Thus, inhibition of MMP-2 activity in the extracellular space is unlikely the result of TIMP-2 dissociation from its complex with MT1-MMP. Consistently, immunoblot analyses of plasma membranes, and surface biotinylation experiments show that the level of surface association of TIMP-2 is independent of MT1-MMP expression. Thus, low affinity binding of TIMP-2 to sites

distinct to MT1-MMP may therefore play a role in regulating MMP-2 activity in the extracellular space generated by the ternary complex.

1C. We have also developed new inhibitors for the gelatinases in collaboration with Dr. Mobashery. Selective inhibition of MMPs is a challenge, because the active sites of MMPs are so similar to one another. Of the over two hundred inhibitors that have been reported for MMPs, a mere handful shows some selectivity for one or more MMP (*4*). We developed a novel strategy for selective irreversible inhibition of gelatinases (MMP-2 and MMP-9). The design process went through a number of iterations. We prepared both peptidic and non-peptidic analogues among these initial sets of compounds. Our efforts ultimately paid off in our development of the first prototype mechanism-based inhibitor for MMPs. The inhibitor, (4-phenoxyphenylsulfonyl)methylthiirane (referred as inhibitor **1** or as SB-3CT in some of our publications), was designed to bind the active sites of gelatinases, MMP-2 and MMP-9, specifically and the thiirane moiety was expected to coordinate to the active-site zinc ion. Inhibitor **1** showed a dual behavior. It served as a mechanism-based inhibitor and as a slow-binding inhibitor, for which the rate constants for the onset of inhibition (k_{on}) and recovery of activity from inhibition (k_{off}) were evaluated. The K_i values were 13.9 $\pm$ 0.4 nM and 600 $\pm$ 200 nM for MMP-2 and MMP-9, respectively, and 100 $\pm$ 11 nM for MT1-MMP. In contrast, the corresponding K_i values for the other MMPs tested, including MMP-3, which did show the slow-binding and mechanism-based inhibition profiles, were at best in the micromolar range. Interestingly, the values for k_{on} (first-order rate constant for the on-set of slow-binding inhibition) were 611- and 78-fold larger (more favorable) for MMP-2 and MMP-9, respectively, than that for MMP-3. We also found that inhibitor **1** inhibited MMP-14 (MT1-MMP) as a simple competitive (reversible) inhibitor (discussed more fully in Specific Aim 1). In addition, the K_i for MMP-14 inhibition was lower than that for MMP-9. Hence, the prototype inhibitor **1** is best for MMP-2 (irreversible and most potent) and is an irreversible MMP-9 inhibitor. In conclusion, we have designed and evaluated the first mechanism-based inhibitor for MMPs, which shows selective and irreversible inhibition of gelatinases. Currently, we are testing inhibitor 1 in animal models of breast cancer metastasis.

2. <u>Studies on MMP-9</u>. (Bioch. Biophys. Res. Comm., 308, 386-395, 2003).

MMP-9 (gelatinase B) is produced in a latent form (pro-MMP-9) that requires activation to achieve catalytic activity. Previously, we showed that MMP-2 (gelatinase A) is an activator of pro-MMP-9 in solution. However, in cultured cells pro-MMP-9 remains in a latent form even in the presence of MMP-2. Since pro-MMP-2 is activated on the cell surface by MT1-MMP in a process that requires TIMP-2, we investigated the role of the MT1-MMP/MMP-2 axis and TIMPs in mediating pro-MMP-9 activation. Full pro-MMP-9 activation was accomplished via a cascade of zymogen activation initiated by MT1-MMP and mediated by MMP-2 in a process that is tightly regulated by TIMPs. We show that TIMP-2 by regulating pro-MMP-2 activation can also act as a positive regulator of pro-MMP-9 activation. Also, activation of pro-MMP-9 by MMP-2 or MMP-3 was more efficient in the presence of purified plasma membrane fractions than activation in a soluble phase or in live cells suggesting that concentration of pro-MMP-9 in the pericellular space may favor activation and catalytic competence.

The studies to identified MMP-9 interacting proteins on the cell surface were unsuccessful and therefore were dropped. We had been working on finding surface proteins that may act as surface binding proteins for MMP-9. Several approaches were used including affinity chromatography using immobilized MMP-9 and phage display. The latter technique was pursued during the last year of the funding period. However, due to the inconsistency of the results we could not pinpoint a specific target of MMP-9 binding. This project was also affected by the sudden departure of Dr. Gupta, who decided to go back to her home country.

3. Reviews

During the granting period, we wrote several reviews on the surface association and regulation of gelatinases (MMP-2 and MMP-9) and MT1-MMP.

1. Hernandez-Barrantes, S., Bernardo, M., Toth, M. and Fridman, R. (2002) Regulation of membrane type-matrix metalloproteinases. Seminars in Cancer Biology, 12, 131-138.

2. Fridman, R. (2003) Surface association of soluble matrix metalloproteinases. In "Surface bound proteolytic enzymes in cancer" Current Topics in Developmental Biology, Vol. 54, pp. 75-99, Eds. S. Zucker and W-T. Chen, Elsevier Science (USA).

3. Fridman, R., Toth, M., Chvyrkova, I., Meroueh, S., and Mobashery, S. (2003) "Cell surface association of MMP-9" Cancer Metastasis Reviews, 22, 153-166.

4. Osenkowski, P, Toth, M. and Fridman R. (2003) Processing, shedding and endocytosis of membrane type-1 matrix metalloproteinase (MT1-MMP). J. Cell. Physiol., in press.

KEY RESEARCH ACCOMPLISHMENTS (2002-2003)

- Characterized the processing of MT1-MMP and described the major enzyme forms
- Characterized the ectodomain shedding of MT1-MMP and describe the major forms
- Characterized the process of activation of pro-MMP-2 by MT1-MMP
- Developed a new approach for gelatinase inhibition using "suicide" inhibitors
- Characterized the activation of pro-MMP-9 by the MT1-MMP/TIMP-2/MMP-2 axis
- Wrote several reviews on MMP surface association and regulation

REPORTABLE OUTCOMES (ALL YEARS)

Manuscripts:

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8. Kotra, L.P., Cross, J.B., Shimura, Y., Fridman, R., Schlegel, H.B., and Mobashery, S. (2001). Insight into the complex process of activation of matrix metalloproteinases. J. Am. Chem. Soc., 123, 3108 -3113.

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21. Liu, X-W., Bernardo, M.M., Fridman, R., and Kim, H-R Choi. (2003) Tissue inhibitor of metalloproteinase-1 (TIMP-1) protects human breast epithelial cells against intrinsic apoptotic cell death via the FAK/PI 3-kinase and MAPK signaling pathway. J. Biol. Chem., 278, 40364-72.

22. Osenkowski, P, Toth, M. and Fridman R. (2003) Processing, shedding and endocytosis of membrane type-1 matrix metalloproteinase (MT1-MMP). J. Cell. Physiol., in press.

Abstracts:

1. Bernardo, M.M., Toth, M., Hernandez-Barrantes, S., Gervasi, D., and Fridman, R. "TIMP-2 Regulation Of Pro-MMP-2 Activation By MT1-MMP." International Meeting on Proteinase Inhibitors and Activators. Strategic Targets for Therapeutic Interventions. University of Oxford, England, UK April 17-20, 2000.

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3. Toth, M. and Fridman, R. "MT-MMP-TIMP interactions regulate enzyme processing and activity on the cell surface." Gordon Research Conference on Matrix Metalloproteinases, Il Ciocco, Italy, May 2001.

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6. Fridman R. "Complex regulation of membrane type-1 matrix metalloproteinase by natural and synthetic MMP inhibitors." 2nd International Conference on Tumor Microenvironment: Progression, Therapy & Prevention. Baden, Austria, June 2002.
7. Ossenkowski, P. Toth, M, Hernandez-Barrantes, S., and Fridman, R. "Structure-function relationship studies of membrane type-1 matrix metalloproteinase." Gordon Research Conference on Proteolytic Enzymes, New Hampshire, July 2002.
8. Presented Abstracts at the two DOD Breast Cancer Research Program Meetings in Washington DC and in Orlando, FL.

Patents:

"Novel mechanism-based Inhibitors for Matrix Metalloproteinases." Inventors: Drs. Shahriar Mobashery and Rafael Fridman, approved May 2000.

Personnel:

Rafael Fridman, Principal Investigator

Marta Toth, Research Associate

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Dave Gervasi, Research Assistant

CONCLUSIONS

1. Pro-MMP-9 is produced in a monomeric and disulfide-bond dimeric form that can bind TIMP-1. These forms were purified to homogeneity and their enzyme kinetics was characterized. The dimeric form of pro-MMP-9 exhibits a slow rate of activation by stromelysin 1 compared to the monomeric form consistent with requirement for conformational changes.

2. The latent forms of MMP-2 and MMP-9 are activated on the tumor cell surface via a cascade of zymogen activation initiated by MT1-MMP and regulated by TIMP-2. Thus, under certain conditions TIMP-2 may act as a positive regulator of pericellular proteolysis and thus contribute to tumor cell invasion.

3. MT1-MMP, one of the major collagenolytic enzymes in breast cancer, is regulated on the cell surface by a complex process of autocatalytic degradation and ectodomain shedding that control the level of enzyme on the cell surface. We characterized the major species resulting from autocatalytic processing and initiated the studies to identify the shedding fragments.

4. Synthetic MMP inhibitors work synergistically with TIMP-2 to stimulate MT1-MMP-dependent activity in the pericellular space. This effect is mediated by the ability of synthetic MMP inhibitors to prevent the autocatalytic processing of MT1-MMP on the cell surface, which results in accumulation of active enzyme. As a consequence, TIMP-2 can form more complexes with MT1-MMP, which act as a "surface receptors" for pro-MMP-2, leading to its activation.

5. Pro-MMP-2 activation by MT1-MMP on the cell surface requires TIMP-2. We have found that TIMP-2 is released with the active MMP-2 and forms a complex that can be detected in the extracellular space. The amount of net active MMP-2 depends on a tight balance between MMP-2, TIMP-2 and MT1-MMP.

6. TIMP-4 cannot support pro-MMP-2 activation by MT1-MMP via formation of a ternary complex. However, TIMP-4 is a potent inhibitor of pro-MMP-2 activation.

7. A novel mechanism-based gelatinase synthetic inhibitor was designed and found to inhibit MMP-2 and MMP-9 in an irreversible manner and with nanomolar affinity.

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APPENDICES

1. Updated CV

2. Brown, S., Bernardo, M. M., Li, Z-H., Kotra, L.P., Tanaka, S., Fridman, R., and Mobashery, S. (2000). Potent and selective mechanism-based inhibition of gelatinases. J. Am. Chem. Soc., 122, 6799-6800.

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Appendix 1

November 2003

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EDUCATION

1977-1978	B.Sc., Biochemistry and Microbiology, Hebrew University of Jerusalem, Jerusalem, Israel.
1979-1981	M.Sc., Developmental Biology, Department of Experimental Medicine and Cancer Research, Hebrew University Medical School, Jerusalem, Israel.
1982-1986	Ph.D., Tumor Biology, Department of Radiation and Clinical Oncology, Hebrew University Medical School, Jerusalem, Israel.
1986-1990	Postdoctoral Fellow, National Institute of Dental Research, NIH.
1990-1991	Visiting Associate, Laboratory of Pathology, NCI, NIH.

PROFESSIONAL AND FACULTY APPOINTMENTS

1991-1992	Staff Scientist, Molecular Oncology Inc., Gaithersburg, MD.
1992-4/2000	Associate Professor, Department of Pathology, School of Medicine Wayne State University, Detroit, MI.
4/2000-Present	Professor, Department of Pathology, School of Medicine Wayne State University, Detroit, MI.
1992-Present	Member, Karmanos Cancer Institute, Detroit, MI. Co-leader, Protease and Cancer Program.

FELLOWSHIPS AND AWARDS

Research Training Fellowship, International Agency for Research on Cancer, World Health Organization (December 1986).

Lady Davis Fellowship for Visiting Professors, Hebrew University, Jerusalem, Israel (April 1997).

EDITORIALSHIP

3/2001-12/2002, Associate Editor, Cancer Research, American Association for Cancer Research
3/2003-present, Associate Editor, Journal of Cellular Physiology

GRANT SUPPORT

ACTIVE

1. PI, NIH, NCI (RO1-CA61986-06) "Regulation of Matrix Metalloproteinase Activation," Total Cost: $1,322,278; July 2000-May 2005, 30% Effort.

2. PI, NIH, NCI (RO1-CA82298) "Surface Binding and Activation of MMP-9 in Tumor Cells," Total Cost: $1,252,647; July 1999-June 2004, 20% Effort.

3. PI, NIH, NCI (RO1-CA100475) "Novel Approach for Inhibition of the MT1-MMP/gelatinase Axis," Total Cost $1,247,132, July 2003-June 2007, 10% Effort.

4. Co-PI, (PI, Dr. Kim) NIH, NCI (RO1-CA89113) "Role of TIMPs in Apoptosis" Total Cost: $1,311,968; July 2002-June 2007. 7% Effort.

5. Co-PI (PI: Michael Cher), DOD Prostate Cancer Program. "Synergy Consortium Targeting New Therapeutics for Lethal Phenotypes of Prostate Cancer." Total budget: $10,000,000 (Multi-institutional). 5% effort.

Previous Grant Funding

1. PI, NIH SBIR (R43 CA56257) "Expression of Enzymes and Inhibitors involved in Metastasis," Total Cost: $50,000, January-July 1992.

2. PI, NCI/NIH Core Grant. "Cancer Center Development Funds (Meyer L. Prentis Comprehensive Cancer Center)," Total Cost: $29,000, October 1992-November 1993.

3. PI, American Cancer Society Institutional Grant. "Biochemical and Biological Characterization of Human Recombinant Type IV Collagenases and TIMPs," Total Cost: $13,000, April 1993-May 1994.

4. PI, Wayne State University Supplemental Research Equipment Fund. "Recombinant Vaccinia Virus Mammalian Cell Expression system," Total Cost: $13,727, April 1993.

5. PI, Wayne State University Supplemental Research Equipment Fund. Total Cost: $26,791, May 1995.

6. PI, Virtual Discovery Grant, Karmanos Cancer Center "Expression and Regulation of Gelatinases and TIMP-2 in Bladder Cancer," Total Cost: $43,191, July 1996-November 1997.

7. Co-PI, Virtual Discovery Grant, Karmanos Cancer Center "Selective and Irreversible Inactivators of Gelatinases" PI: Dr. Sharhiar Mobashery, Total Cost: $43,000, July 1996-November 1997.

8. PI, Postdoctoral Fellowship, Wayne State University (Dr. Matthew Olson), Total Cost: $20,000, January 1996-December 1997.

9. PI, Department of Defense, US Army (DAMD17-94-J-4356) "Role of Proteases in Breast Cancer Progression," Total Cost: $814,994, July 1994-June 1998.

10. PI, NIH (RO1-CA61986-05) "Regulation of Matrix Metalloproteinase Activation," Total Direct Cost: $800,000; July 1995-May 2000, 30% Effort.

11. Co-PI, (PI, Dr. Berk) NIH (RO1-EYI11757) "Role of TIMPs in P. Aeruginosa Corneal Infections," Total Direct Cost: $434,915, July 1998-June 2001. Consultant.

12. Co-PI (PI, Dr. Michael Cher), DOD Prostate Cancer Research Program, IDEA grant (PC991065), "A Novel Approach to Manipulate the Human Bone Microenvironment in Prostate Cancer: Prevention of Bone Matrix Degradation by Inhibition of Matrix Metalloproteinases," Total Direct Cost: $75,000, January 2000-December 2001, 5% Effort.

13. Ph.D. Advisor (Graduate Student PI: Sonia Hernandez) DOD Breast Cancer Research Program, Predoctoral Fellowship "Extracellular Matrix Regulation of Membrane Type 1-Matrix Metalloproteinase (MT1-MMP) and Matrix Metalloproteinase-2 (MMP-2) in Human Breast Fibroblasts," Total Direct Cost: $65,670, July1999-September 2002.

14. PI, DOD Breast Cancer Research Program, Academic Award (DAMD17-99-1-9940) "Cell Surface Regulation of Matrix Metalloproteinases in Breast Cancer Cells," Total Direct Cost: $443,297; July 1999-August 2003, 20% Effort.

<u>Awarded but declined</u>

1. PI, NIH (RO1 CA64788-01) "Proteases and Breast Stromal-Epithelial Interactions," Total Cost: $666,309, December 1994. Awarded but declined to accept US Army grant (DAMD17-94-J-4356).

PUBLICATIONS (in chronological order)

1. **Fridman, R.**, Gelfand, T., Weiss, D.W. and Doljanski, F. (1984). Patterns of fibronectin deposition in normal and neoplastic fibroblasts and mammary tissue. Int. J. of Tissue Reactions, 4, 291-301.

2. Ovadia, H., Lubetzki-Korn, I., Brenner, T., Abramsky, O., **Fridman, R.** and Vlodavsky, I. (1984). Adult rat oligodendrocytes grown *in vitro* upon an extracellular matrix have the ability to proliferate. Brain Research, 322: 93-100.

3. Eldor, A., **Fridman, R.**, Vlodavsky, I., Hy-Am, E., Fuks, Z. and Panet, A. (1984). Interferon enhances prostacyclin production by vascular endothelial cells. J. Clin. Invest., 73, 251-257.

4. Abramsky, O., Lubetzki-Korn, I., **Fridman, R.**, Vlodavsky, I., and Ovadia, H. (1984). Culture of isolated mature oligodendrocytes on extracellular matrix. Symposia of the Giovanni Lorenzini Foundation, Developmental Neuroscience: Physiological, Pharmacological and Clinical Aspects. Ed. F. Ciagli, E. Jacobini, and R. Paoletti, Vol. 20, pp. 351-354, Elsevier Press.

5. **Fridman, R.**, Fuks, Z., Ovadia, H. and Vlodavsky, I. (1985). Differential structural requirements for the induction of cell attachment, proliferation and differentiation by the extracellular matrix. Exp. Cell Res., 157, 181-194.

6. **Fridman, R.**, Alon, Y., Doljanski, F., Fuks, Z. and Vlodavsky, I. (1985). Cell interactions with the extracellular matrices produced by endothelial cells and fibroblasts. Exp. Cell Res., 158, 461-476.

7. Heyns, A., Eldor, A., Vlodavsky, I., Kaiser, N., **Fridman, R.** and Panet, A. (1985). The antiproliferative effect of interferon and the mitogenic activity of growth factors are independent cell cycle events. Exp. Cell Res. 161, 297-306.

8. Cohen, I.R., Lider, O., Baharav, E., Hardan, I., Miller, T., Bar-Ner, M., Naparstek, Y., **Fridman, R.**, and Vlodavsky, I. (1986). Regulation of experimental autoimmunity and allograft rejection by heparins that inhibit T lymphocyte heparanase. Proc. 1st IUIS Conf. on Clinical Immunol., Toronto, Canada, Elsevier Press.

9. **Fridman, R.**, Lider, O., Naparstek, Y., Vlodavsky, I., Fuks, Z. and Cohen, I.R. (1987). Soluble antigen induces T lymphocytes to secrete an endoglycosidase that degrades the heparan sulfate moiety of subendothelial extracellular matrix. J. Cell. Physiol., 130, 85-92.

10. Vlodavsky, I., Folkman, I., Sullivan, R., **Fridman, R.**, Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M. (1987). Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. Proc. Natl. Acad. Sci. USA., 84, 2292-2296.

11. Vlodavsky, I., Eldor, A., Bar-Ner, M., **Fridman, R.**, Cohen, I.R., and Klagsbrun, M. (1987). Heparan sulfate degradation in tumor cell invasion and angiogenesis. Adv. Exp. Med. Biol., 233, 201-210.

12. Vlodavsky, I., **Fridman, R.**, Sullivan, R., Sasse, J., and Klagsbrun, M. (1988). Aortic endothelial cells synthesize basic fibroblast growth factor, which remains cell associated, and platelet-derived growth factor, which is secreted. J. Cell. Physiol. 181, 142-148.

13. Vlodavsky, I., Ishai-Michaeli, R., Bar-Ner, M., **Fridman, R.**, Horowitz, A., Fuks, Z., and Biran, S. (1988). Involvement of heparanase in tumor metastasis and angiogenesis. Israel J. of Med., 24, 464-470.

14. Albini, A., Aukerman, S.L., Ogle, R.C., Noonan, D.M., **Fridman, R.**, Martin, G.R., and Fidler, I.J. (1988). The *in vitro* invasiveness and interactions with laminin of K-1735 melanoma cells. Evidence for different laminin-binding affinities in high and low metastatic variants. Clinical Exp. Metastasis, 4, 437-451.

15. Bonfil, R.D., Reddel, R.R., Ura, H., Reich, R., **Fridman, R.**, Harris, C.C., and Klein-Szanto, A.J.P. (1989). Invasive and metastatic potential of a Ha-ras transformed human bronchial epithelial cell line. J. Natl. Can. Inst., 81, 587-594.

16. Penno, M.B., Passaniti, A., **Fridman, R.**, Hart, G.W., Jordan, C., Kumar, S., and Scott, A.F. (1989). *In vitro* galactosylation of a 110-kDa glycoprotein by an endogenous cell surface galactosyltransferase correlates with the invasiveness of adrenal carcinoma cells. Proc. Acad. Natl. Sci. USA, 86, 6057-6061.

17. Ovadia, H., Lubetzki-Korn, I., **Fridman, R.**, Vlodavsky, I., and Abramsky, O. (1989). Use of naturally produced basement membrane-like extracellular matrix for the growth and maintenance of isolated oligodendrocytes. In A dissection and tissue culture manual of the nervous system (Shahar, de Vellis, Vernadakis, and Haber, eds.) pp.153-156, Alan R. Liss, Inc. New York.

18. Bonfil, R.D., Momiki, S., **Fridman, R.**, Reich, R., Reddel, R., Harris, C.C., and Klein-Szanto, A.J.P. (1990). Enhancement of the invasive ability of a transformed human bronchial epithelial cell line by TPA and diacylglycerol. Carcinogenesis, 10, 2335-2338.

19. **Fridman, R.**, Lacal, J.C., Reich, R., Bonfil R.D., and Ahn, C. (1990). Differential effect of phorbol ester on the *in vitro* invasiveness of malignant and non-malignant human fibroblast cells. J. Cell. Physiol., 142, 55-60.

20. **Fridman, R.**, Scott, A.F., Muller, D., Reich, R., and Penno, M.B. (1990). The role of cell adhesion and migration in the *in vitro* invasiveness of mouse adrenal carcinoma cells. Invasion and Metastasis, 10, 208-224.

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25. Kanemoto, T., Martin, G.R., Hamilton, T., and **Fridman, R.** (1991). Effect of synthetic peptides and protease inhibitors on the interaction of a human ovarian cancer cell line (NIH:OVCAR-3) with a reconstituted basement membrane (Matrigel). Invasion and Metastasis, 11, 84-92.

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36. Schnaper, H. W., Grant, D.S., Stetler-Stevenson, W.G., **Fridman, R.**, D'Orazi, G., Murphy, A.N., Bird, R.E., Hoyhtya, M., Fuerst, T.R., French, D., Quigley, J.P., and Kleinman, H.K. (1993). Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis *in vitro*. J. Cell. Physiol., 156, 235-246.

37. Liebman, J.M., Burbelo, P.D., Yamada, Y., **Fridman, R.**, Kleinman, H.K. (1993). Altered expression of basement-membrane components and collagenases in ascitic xenografts of OVCAR-3 ovarian cancer cells. Int. J. Cancer 55, 102-109.

38. Hoyhtya, M., **Fridman, R.**, Komarek, D., Porter-Jordan, K., Stetler-Stevenson, W.G., Liotta, L.A., and Liang, C-M. (1994). Immunohistochemical localization of matrix metalloproteinase-2 and its specific inhibitor TIMP-2 in neoplastic tissues with monoclonal antibodies. Int. J. Cancer, 56, 500-505.

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41. Ochieng, J., **Fridman, R.**, Nangia-Makker, P., Liotta, L.A., Stetler-Stevenson, W.G., and Raz, A. (1994). Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. Biochemistry, 33, 14109-14114.

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44. Belloc, C., Lu, H., Soria, C., **Fridman, R.**, Legrand, Y., And Menashi, S. (1995). The effect of platelets on invasiveness and protease production of human mammary tumor cells. Int. J. Cancer, 60, 413-417.

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PATENTS

1. "Method and composition for growing tumors from few cells." The patent describes the use of Matrigel for growing human tumor cells in animal models. Submitted March 30, 1990. Inventors: Drs. Rafael Fridman, Hynda Kleinman, and George Martin.

2. "Novel mechanism-based Inhibitors for Matrix Metalloproteinases." Inventors: Drs. Shahriar Mobashery and Rafael Fridman, approved May 2000.

RESEARCH SERVICE

Study Sections and Grant Reviewer

1994-1996-2002, Member, Pathobiology Study Section, Breast Cancer Program, US Army
6/1996, Ad Hoc Reviewer, Pathobiochemistry Study Section, NCI/NIH
5/1997, Member, Cancer Study section, Tobacco-Related Dis. Res. Program, UCSF
5/1998, Member, Pathobiology Study Section, Prostate Cancer Program, US Army
11/1998, Knowledge Harvest Program, Breast Cancer Program, US Army
4/1999, Ad Hoc Reviewer, Special Study Section, NCI, NIH
3/2000, Ad Hoc Reviewer, Program Project Special Study Section, NCI, NIH
9/2001, Ad Hoc Reviewer, Program Project Special Study Section, NCI, NIH
10/2001, Ad Hoc Reviewer, Program Project Special Study Section, NHLBI, NIH
3/2003, Member, Manpower & Training Study Section (NCI-I), NCI, NIH

American Cancer Society, local Chapter.
Wayne State University Internal Research and Travel Grants.
Israel Academy of Sciences.
Wellcome Trust Foundation.
Medical Research Council of Canada.
Association for International Cancer Research

Ad Hoc Journal Reviewer

More than 400 manuscripts reviewed since 1993 for the following journals:

Science, Proceedings of the National Academy of Sciences, Cancer Research, Journal of Biological Chemistry, Journal of the National Cancer Institute, Cancer, Journal of Clinical and Experimental Metastasis, Invasion and Metastasis, International Journal of Cancer, Journal of Cellular Physiology, Experimental Cell Research, American Journal of Obstetrics and Gynecology, American Journal of Pathology, Experimental Hematology, American Journal of Physiology, Biochimica et Biophysica Acta, Journal of Immunological Methods, Biochemistry and Cell Biology, British Journal of Cancer, Journal of Clinical Investigation, Journal of Cell Science, Cancer Letters, Oncogene and Journal of Molecular Biology.

Other Research Services

1. Departmental Committees

Pathology Internal Review Committee, 1995.
Pathology Salary Committee, 1995-2002.
Pathology Equipment Committee, 1993-1997.
Pathology Promotion and Tenure Committee, 2000-2002

2. University Committees

Karmanos Cancer Center, Member, Protease Section, 1992-present, Deputy Director
Academic Senate, 1994-1997.
Research Committee, School of Medicine, 1995-1998.
Research Committee, Wayne State University, 1995-1998.

3. Extramural Research Activities

Provide reagents (enzymes, inhibitors, antibodies, cells) to investigators worldwide in the area of Matrix Metalloproteinases.

TEACHING

1. Formal Courses

1. Medical Students:

Instructor, Year II, Pathobiology Laboratory Course, 1993-1996.

2. Graduate Students:

Fundamentals of Cancer Biology (CB7210), Lecturer.
Seminars in Cancer Biology (CB 7890), Director.
Seminars in Pathology (PTH 760), Director.
IBS Cell Biology (IBS 7020), Lecturer

2. Graduate Students and Postdoctoral Fellows and Mentorship

1. Undergraduate:

Adam Treitman (1995) (MSc, Basic Medical Sciences), Practical Laboratory Training, Advisor.
Andrew Kontos (1996) (MSc, Medical Sciences), Essay Dissertation Advisor.
Moshira Ghabrial (1997) (MSc, Medical Sciences), Essay Dissertation Advisor.
Yuval Raz (1997) (MSc, Medical Sciences), Essay Dissertation Advisor.
Jinqiu Li, Pathology (2001) (MSc, Medical Sciences), Essay Dissertation Advisor
Ari Konheim (2002) DOD Summer Research Program Fellowship

2. Graduate (Ph.D.):

Zhong Dong, Pathology, (1997-1999), Thesis Advisor.
Sonia Hernandez, Cancer Biology, (1997-2001), Thesis Advisor
Pamela Osenkowski, Cancer Biology (2001-present), Thesis Advisor
Sun Qing, Pathology (2003-present), Thesis Advisor
Jinah Cho, Pathology (2003-present), Thesis Advisor

3. Postdoctoral Fellows:

Daniel Peña (8/93-12/94)
Karen Kernacki (3/95-7/96)
Matthew Olson (1/96-12/97)
Maria Yurkova (12/96-7/98)
Yoishiro Shimura (9/99-9/00)
Irina Schvrykova (10/00-9/03)

4. Residents and Physicians (Mentorship):

Dr. Fernando Bianco, Urology (7/96-7/97)
Dr. Mary Kosir, Surgery (95/96)

3. Other Teaching Related Services

Graduate School Faculty, Member, 1992-present
Pathology Graduate Committee 1993-1998.
Committee for the Assessment of Graduate Education (CAGE), 1996-1997.
Postdoctoral Enhancement Committee, 1996-1997.
Interdisciplinary Biomedical Sciences Executive Committee, 1998-present.
Executive Committee of the Graduate Program in Cancer Biology, 1999-present

INVITED PRESENTATIONS:

1991 Lake Ontario Metastasis Society, Montreal, Canada.
1992 Sloan Kettering Cancer Center, New York, NY.
1993 Beaumont Hospital, Royal Oak, MI.
Upjohn Co., March 1993, Kalamazoo, MI.
1994 International Conference on Proteases in Inflammation and Cancer, Lubljana, Slovenia.
Hebrew University, Hadassah Hospital, Jerusalem, Israel.
1995 Department of Chemistry, Wayne State University

1996 AACR Conference on Proteases and Protease Inhibitors in Cancer, Panama City Beach, FL.
1997 Sixth International Symposium on Cancer, Seoul National University, Seoul, Korea.
Matrix Metalloproteinases, Gordon Research Conference, Proctor Academy, Andover, NH.
Frontiers in Bladder Cancer Treatment, Birmingham, MI.
Beamount Hospital, Royal Oak, MI.
University of Michigan, Ann Arbor, MI.
1998 Unraveling the Biological and Pathological Function of Proteolytic Enzymes, Wayne State University, Detroit, MI.
INSERM, Paris, France.
State University of New York, Stony Brook, NY.
1999 Annual Forbeck Forum on Tumor Invasion and Metastasis, February 7-10, 1999, Ein-Gedi, Israel.
2000 Argentinean Association for Clinical Research, August, 2000, Buenos Aires Argentina.

Arkansas Cancer Research Center, Little Rock, AK.
Royal London School of Medicine and Dentistry, London, UK
Mayo Clinic, Cancer Center, Rochester, MN.
Department of Cell Biology, University of Virginia, Charlottesville, VA.

2001 International Meeting on Surface Proteases in Cancer, May 2001, Palermo, Italy.
Beaumont Hospital, Royal Oak, MI.

2002 Henry Ford Hospital, Detroit, MI
Imclone, NY, NY.
Department of Structural Biology, Weizmann Institute, Rehoboth, Israel.
Oncoloy, Hadassah Hospital, Jerusalem, Israel
Feist-Weiller Cancer Center, LSU-HSC, Shreveport, LA.
Meharry Medical College, Nashville, TN.
INSERM, Paris, France.
2nd International Meeting on Tumor Microenvironment: Progression, Therpy and Prevention, June 2002, Baden, Austria.
9th International Congress of the Metastasis Research Society, September 2002, Chicago, USA
Tumor Invasion, Metastasis and Angiogenesis Mini-Symposium, R. H. Laurie Comprehensive Cancer Center, Northwestern University, September 2002, Chicago, USA.

2003 Department of Anatomy and Cell Biology, Wayne State University.
University of British Columbia, Vancouver, Canada.
Session Chair and speaker, Gordon Conference on Matrix Metalloproteinase, August 2003.

Appendix 2

Potent and Selective Mechanism-Based Inhibition of Gelatinases

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Potent and Selective Mechanism-Based Inhibition of Gelatinases

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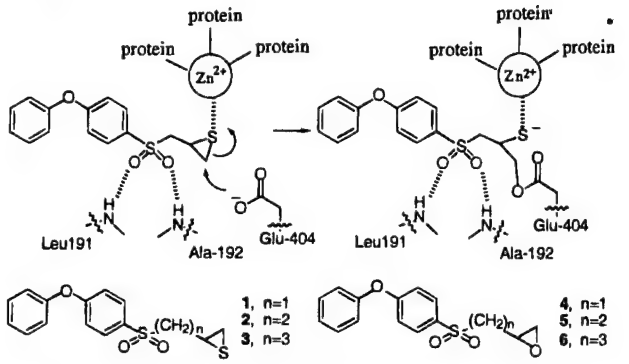
Specific interactions of cells within the extracellular matrix are critical for the normal function of the organism. Alterations of the extracellular matrix are carried out by a family of zinc-dependent endopeptidases called matrix metalloproteinases (MMPs) in various cellular processes such as organ development, ovulation, fetus implantation in the uterus, embriogenesis, wound healing, and angiogenesis.[1,2] Gelatinases, collagenases, stromelysins, membrane-type MMPs, and matrilysin comprise the five major groups of MMPs, of which at least 26 members have been identified in humans to date. The activities of MMPs in physiological conditions are strictly regulated by a series of complicated zymogen activation processes and inhibition by the protein tissue inhibitors of metalloprotainases ("TIMPs").[2,3] Excessive MMP activity has been implicated in cancer growth, tumor metastasis and angiogenesis, arthritis, connective tissue diseases, inflammation, and cardiovascular and autoimmune diseases.[1,2,4] Due to the potential therapeutic value of MMP inhibitors for these conditions, synthetic inhibitors of MMPs are highly sought.[5,6] All the known inhibitors for MMPs take advantage of chelation to the active site zinc ion for inhibition of activity. The known MMP inhibitors usually suffer from toxicity to hosts.[5a,c,7] Besides the issue of undesirable side effects, the design of MMP inhibitors has been complicated by only low levels of specificity among members of the MMP family, which hampers our ability to target specific MMPs in each pathological condition. We describe herein the first mechanism-based inhibitor for MMPs, a novel concept for the selective inhibition of these enzymes. We show that our inhibitor rivals the action of TIMPs in its efficacy in inhibition of MMPs.

Increased level of activity for human gelatinases, MMP-2 and MMP-9, has been implicated in the process of tumor metastasis and angiogenesis.[8] As a result, we have been interested in the selective inhibition of these two key MMPs. For this purpose, we have resorted to the design of mechanism-based inhibitors

Scheme 1



(also known as "k_{cat} inhibitors" or "suicide substrates"). This type of inhibitor has the potential to impart high selectivity in inhibition of closely related enzymes, such as MMPs.[9] Our strategy for mechanism-based inhibition of MMPs by compound **1** is depicted in Scheme 1. The strategy envisions that coordination of the thiirane with the active-site zinc ion would activate it for modification by a nucleophile in the enzyme active site. The biphenyl moiety in compounds **1**−**6** would fit in the $P_{1'}$ subsite of gelatinases, which is a deep hydrophobic pocket.[10,11] Energy-minimized complexes of MMP-2 and MMP-9[12] with compound **1** indicated that the biphenyl group would fit in the active site analogously to the same group in reversible inhibitors of MMP-2 and MMP-9.[6] This binding mode would bring the sulfur of the thiirane in **1** into the coordination sphere of the zinc ion. The models indicated that the thiirane moiety in compounds **2** and **3**, with longer carbon backbones, would not be able to coordinate with the zinc ion, but would fit in an extended conformation in the active site.

Scheme 2 shows the synthetic route for compounds **1**−**6**. 4-Phenoxythiophenol **10** was prepared from the commercially available 4-phenoxyphenol **7** via a three-step procedure described by Newman and Karnes for a related system.[13] Subsequent alkylation of **10** with allyl bromide, 4-bromo-1-butene, and 5-bromo-1-pentene, respectively, led to the sulfanyl compounds **11**−**13** in good yields. Epoxidation of **12** and **13** with mCPBA proceeded in 2−3 days, but that for **11** took 7 days and required an excess of mCPBA. Finally, conversion of the epoxides **4**−**6** to their corresponding thiirane derivatives **1**−**3**, respectively, was accomplished by the treatment of each epoxide with ammonium thiocyanate. Although the thiiranes **2** and **3** were isolated in high yields (93 and 85%, respectively), thiirane **1** could only be recovered in a poor 14% yield.

Compounds **1**−**6** were evaluated with MMPs.[14] Whereas inhibitors **2**−**6** showed either no inhibition or relatively poor

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Scheme 2

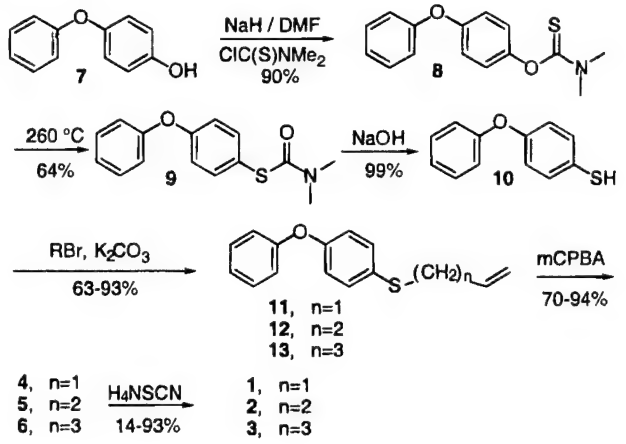

Table 1. Kinetic Parameters for Inhibition of MMPs by the Synthetic Inhibitor

	$10^{-4}k_{on}$ ($M^{-1}s^{-1}$)	$10^{3}k_{off}$ (s^{-1})	K_i (μM)
Inhibitor 1			
MMP-2	11 ± 1	1.8 ± 0.1	0.0139 ± 0.0004
MMP-9	1.4 ± 0.3	7.1 ± 0.5	0.6 ± 0.2
MMP-3	0.018 ± 0.004	5.5 ± 0.4	15 ± 6
MMP-7			96 ± 41
MMP-1			206 ± 60
TIMP-1[18]			
MMP-2	4.4 ± 0.1	1.3 ± 0.2	0.029 ± 0.005
MMP-9	5.2 ± 0.1	1.2 ± 0.2	0.024 ± 0.004
TIMP-2[18]			
MMP-2	3.3 ± 0.1	0.8 ± 0.1	0.023 ± 0.004
MMP-9	2.2 ± 0.1	1.3 ± 0.2	0.058 ± 0.007

inhibition of the MMPs (K_i values of micromolar at best; see Supporting Information), the behavior of inhibitor **1** was different. Inhibitor **1** showed a dual behavior. It served as a mechanism-based inhibitor with a partition ratio of 79 ± 10 (i.e., k_{cat}/k_{inact}) for MMP-2 and of 416 ± 63 for MMP-9.[15] Furthermore, it also behaved as a slow-binding inhibitor, for which the rate constants for the on-set of inhibition (k_{on}) and recovery of activity from inhibition (k_{off}) were evaluated (Table 1). It would appear that coordination of the thiirane with the zinc ion (as seen in the energy-minimized computational models; Scheme 1) would set in motion a conformational change, which is presumed from the slow-binding kinetic behavior. The kinetic data fit the model for slow-binding inhibition.[16] Covalent modification of the enzymes ensued this conformational change. We incubated inhibitor **1** with MMP-2 to the point that less than 5% activity remained. This inhibitor–enzyme complex was dialyzed over 3 days, which resulted in recovery of approximately 50% of the activity. This observation is consistent with modification of the active site Glu-404, via the formation of an ester bond, which is a relatively labile covalent linkage.[17]

We observe selectivity in inhibition of gelatinases by inhibitor **1**. The K_i values are 13.9 ± 0.4 and 600 ± 200 nM for MMP-2 and MMP-9, respectively. In contrast, the corresponding K_i values for the other MMPs tested, including MMP-3, which does show the slow-binding mechanism-based inhibition profile, are in the micromolar range. Interestingly, the values for k_{on} are 611- and 78-fold larger for MMP-2 and MMP-9, respectively, than that for MMP-3. Collectively, these kinetic parameters make inhibitor **1** a potent and selective inhibitor for both MMP-2 and MMP-9, more so for MMP-2. We have determined previously that two molecules of either TIMP-1 or TIMP-2 bind to activated MMP-2 and MMP-9.[18] One binding event is high affinity and would appear physiologically relevant, whereas the second binding event takes place with relatively lower affinity (micromolar).[18] Inhibition of MMP-2 and MMP-9 by TIMP-2 and TIMP-1, respectively, also follows slow-binding kinetics. The kinetic parameters for these interactions at the high affinity site are listed in Table 1. We find it noteworthy that the kinetic parameters for the slow-binding component of inhibition of MMP-2 and MMP-9 by inhibitor **1** (k_{on} and k_{off}) approach closely the same parameters for those of the TIMPs.[18]

We have outlined in this paper a novel example for potent inhibition of human gelatinases by the small-molecule inhibitor **1**, which follows both slow-binding and mechanism-based inhibition in its kinetic profile. This compound appears to behave similarly to TIMP-2 and TIMP-1 in the slow-binding component of inhibition. Furthermore, the inhibitor also exhibits a covalent mechanism-based behavior in inhibition of these enzymes. The selectivity that inhibitor **1** displays (in both affinities and the modes of inhibition) among the other structurally similar MMPs is noteworthy and should serve as a paradigm in the design of inhibitors for other closely related enzymes in the future.

Acknowledgment. This work was supported by grants DAMD17-97-1-7174 from the US Army (to S.M.) and CA-61986 and CA-82298 from the NIH (to R.F.). We are indebted to Dr. Paul Cannon and Dr. W. Parks for the gifts of human MMP-3 and MMP-1, respectively.

Supporting Information Available: Detailed procedures for syntheses and kinetic determinations are provided (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA001461N

(14) Homogeneous preparations of MMPs were used in our studies. Recombinant human MMP-2 and MMP-9 were prepared as described previously (see Supporting Information). Representative members of the other classes of MMPs, such as stromelysin 1 (MMP-3), matrilysin (MMP-7), and collagenase-1 (MMP-1), were used in our studies.

(15) The partition ratio indicates that there is turnover of the thiirane for each covalent inhibition of the enzyme. The partition ratios were relatively low, such that given the quantities of the enzymes available to us, we were not able to isolate and characterize the product of this turnover.

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(17) The time-dependent loss of activity is not merely due to the slow-binding behavior. For instance, for a k_{off} of 2×10^{-3} s^{-1} (the values are not very different from one another in Table 1) the half-time for recovery of activity ($t_{1/2}$) is calculated at just under 6 min. The fact that 50% of activity still did not recover after dialysis over 3 days strongly argues for the covalency of enzyme modification.

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Appendix 3

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Complex Pattern of Membrane Type 1 Matrix Metalloproteinase Shedding

REGULATION BY AUTOCATALYTIC CELL SURFACE INACTIVATION OF ACTIVE ENZYME*

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Membrane type 1 matrix metalloproteinase (MT1-MMP) is a type I transmembrane MMP shown to play a critical role in normal development and in malignant processes. Emerging evidence indicates that MT1-MMP is regulated by a process of ectodomain shedding. Active MT1-MMP undergoes autocatalytic processing on the cell surface, leading to the formation of an inactive 44-kDa fragment and release of the entire catalytic domain. Analysis of the released MT1-MMP forms in various cell types revealed a complex pattern of shedding involving two major fragments of 50 and 18 kDa and two minor species of 56 and 31–35 kDa. Protease inhibitor studies and a catalytically inactive MT1-MMP mutant revealed both autocatalytic (18 kDa) and non-autocatalytic (56, 50, and 31–35 kDa) shedding mechanisms. Purification and sequencing of the 18-kDa fragment indicated that it extends from Tyr^{112} to Ala^{255}. Structural and sequencing data indicate that shedding of the 18-kDa fragment is initiated at the Gly^{284}-Gly^{285} site, followed by cleavage between the conserved Ala^{255} and Ile^{256} residues near the conserved methionine turn, a structural feature of the catalytic domain of all MMPs. Consistently, a recombinant 18-kDa fragment had no catalytic activity and did not bind TIMP-2. Thus, autocatalytic shedding evolved as a specific mechanism to terminate MT1-MMP activity on the cell surface by disrupting enzyme integrity at a vital structural site. In contrast, functional data suggest that the non-autocatalytic shedding generates soluble active MT1-MMP species capable of binding TIMP-2. These studies suggest that ectodomain shedding regulates the pericellular and extracellular activities of MT1-MMP through a delicate balance of active and inactive enzyme-soluble fragments.

Release of the extracellular portion of type I transmembrane proteins, referred to as ectodomain shedding, has been established as a major regulatory mechanism to control the activity of a variety of membrane-bound proteins on the cell surface (1). Recent evidence suggests that ectodomain shedding is also characteristic of the membrane type matrix metalloproteinases (MT-MMPs),[1] a subfamily of membrane-anchored MMPs by means of a transmembrane domain or a glycosylphosphatidylinositol anchor (2, 3). The MT-MMPs are major mediators of proteolytic events on the cell surface, including turnover of extracellular matrix components (4, 5), cleavage of various surface adhesion receptors (6–8), and initiation of zymogen activation cascades (9, 10). Uncontrolled MT-MMP activity contributes to abnormal development (11) and is a key determinant in cancer metastasis and tumor angiogenesis (12–14). To control the extent of pericellular activity, the MT-MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs), a family of natural protein MMP inhibitors. In addition, MT-MMPs have a unique regulatory mechanism in which the active enzyme undergoes a series of processing steps, either autocatalytic (15–17) or mediated by other proteases (18), that regulate the activity and nature of the enzyme species at the cell surface and at the pericellular space. Previous studies have shown that active MT1-MMP is autocatalytically processed on the cell surface to an inactive membrane-tethered ~44-kDa species lacking the entire catalytic domain (17). This processing is inhibited by TIMP-2, TIMP-4, and synthetic MMP inhibitors consistent with being an intermolecular autocatalytic event (19, 20). Inhibition of MT1-MMP processing induces accumulation of the active enzyme on the cell surface, and, as a consequence, net MT1-MMP-dependent proteolysis is enhanced. Indeed, we have shown that, under certain conditions, inhibition of MT1-MMP autocatalysis by synthetic MMP inhibitors enhances pro-MMP-2 activation by MT1-MMP in the presence of TIMP-2 (20, 21). Thus, although the presence of inhibitors will stabilize MT1-MMP on the cell surface, the absence or reduced levels of inhibitors will facilitate autocatalysis. As a membrane-anchored protein, the autocatalytic processing of active MT1-MMP on the cell surface raises questions as to the fate of the ectodomain and its functional consequences. Accumulating evidence suggests that catalytic domain shedding may represent a general characteristic of several members of the MT-MMP family, and both autocatalytic and non-autocatalytic mechanisms of shedding have been described, *e.g.* autocatalysis was implicated in the shedding of

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[1] The abbreviations used are: MT-MMP, membrane type matrix metalloproteinase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; mAb, monoclonal antibody; pAb, polyclonal antibody; ConA, concanavalin A; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

MT1-MMP in cells transfected to overexpress MT1-MMP (22). Other studies reported shedding of MT1-MMP from a breast carcinoma cell line after treatment with concanavalin A (ConA) (23–25), which was not inhibited by TIMP-2 (23); therefore, autocatalysis could not be involved. MT5-MMP sheds its catalytic domain in a process that appears to be mediated by a pro-convertase that removes the ectodomain intracellularly (26). Pre-mRNA splicing was reported to be involved in the generation of a soluble form of MT3-MMP, which retained catalytic activity and sensitivity to TIMP-2 inhibition (27). Thus, although different mechanisms of shedding may exist, collectively, these data suggest a unique property of MT-MMPs: the ability to generate soluble fragments by a process of ectodomain shedding, which may possess important functional consequences for pericellular proteolysis in normal and malignant processes. Here we have identified the major soluble forms of MT1-MMP and characterized the major autocatalytic fragment. We demonstrated that the autocatalytic shedding mechanism of MT1-MMP is likely to have evolved to terminate MT1-MMP-dependent proteolysis by hydrolyzing the enzyme at specific and vital sites.

EXPERIMENTAL PROCEDURES

Cell Culture—The characteristics and culture conditions of nonmalignant monkey kidney epithelial BS-C-1 (CCL-26) (28), human fibrosarcoma HT-1080 (CCL-121) (29), human fibroblasts HFL1 (CCL-153) (29), and human breast carcinoma MDA-MB-231 (HTB-26) (30) cells have been previously described. Human glioblastoma U-87 MG (HTB-14) cells, obtained from the American Tissue Culture Collection (ATCC), and immortalized homozygous *Timp2* (−/−) mutant mouse fibroblasts, a gift from Dr. P. Soloway (Roswell Park Cancer Institute, Buffalo, NY) (20, 31), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. The *Timp2* (−/−) mutant cells were stably transfected to express full-length human MT1-MMP using an MT1-MMP expression vector (P_6R-MT1-MMP) with hygromycin resistance (a gift from Dr. G. Goldberg, Washington University, St. Louis, MO). A stable hygromycin-resistant clone designated *Timp2* (−/−)-MT1 was selected for further studies.

Recombinant Vaccinia Viruses—The production of the recombinant vaccinia virus (vTF7–3) expressing bacteriophage T7 RNA polymerase has been described (32). Recombinant vaccinia viruses expressing pro-MMP-2 (vT7-GELA), MT1-MMP (vT7-MT1), TIMP-2 (vSC59-T2), or TIMP-1 (vT7-T1) were obtained by homologous recombination as previously described (17, 28, 33).

Recombinant Proteins, Synthetic MMP Inhibitors, and Antibodies—Human recombinant pro-MMP-2, TIMP-2, and TIMP-1 were expressed in HeLa cells and purified to homogeneity, as previously described (34). Human TIMP-4 was a generous gift from Dr. C. Overall (University of British Columbia, Vancouver, Canada). A recombinant catalytic domain of human MT1-MMP (Tyr^{112} to Gly^{288}) designated MT1-MMP_{cat} was purchased from Calbiochem. Marimastat (BB-2516) was obtained from British Biotech (Annapolis, MD) (35). SB-3CT was produced and characterized as described (36). The anti-TIMP-2 monoclonal antibody (mAb) CA-101 (33), the rabbit polyclonal antibody (pAb) 437 to the hemopexin-like domain of MT1-MMP (17), and the rabbit pAb to human TIMP-1 have been previously described (37, 38). The rabbit pAb 160 to the catalytic domain of MT1-MMP (25) was a generous gift from Dr. A. Sang (Florida State University, Tallahassee, FL). The mAb LEM-2/15 to the catalytic domain of human MT1-MMP has been described (39).

Cell Treatments and Metabolic Labeling—HT1080, MDA-MB-231, U-87 and HFL1 cells were grown to 80% confluence in 150-mm tissue culture dishes and then incubated (16 h, 37 °C) with serum-free DMEM (15 ml/dish) supplemented with or without 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or 10 μg/ml concanavalin A (ConA) (Sigma). In some experiments, these cell lines and the *Timp2* (−/−)-MT1 cells were metabolically labeled (6–16 h, 37 °C) with 70–100 μCi/ml [^{35}S]methionine (PerkinElmer Life Sciences) in starving medium (DMEM without methionine supplemented with 25 mM HEPES).

Cell Surface Biotinylation—HT1080 cells in six-well plates were untreated or treated with 100 nM TPA or 10 μg/ml ConA in 1 ml of serum-free medium overnight. The cells were rinsed with cold phosphate-buffered saline containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then biotinylated with 0.5 mg/ml sulfo-NHS-biotin as described (38). The cells were lysed with 0.5 ml/well harvest buffer (0.5% SDS, 60 mM Tris/HCl, pH 7.5, 2 mM EDTA) and boiled. The lysates were supplemented with 2.5% Triton X-100 (final concentration) followed by addition of either pAb 437 or pAb 160/Protein A-agarose beads. After a 12-h incubation period (4 °C), the beads were washed three times with harvest buffer supplemented with 2.5% Triton X-100 (final concentration) followed by one wash with collagenase buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35). The immunoprecipitates were eluted with Laemmli SDS sample buffer (40), boiled, and resolved by reducing 8–16% SDS-PAGE, followed by transfer to a nitrocellulose membrane. The biotinylated MT1-MMP forms were detected with streptavidin-horseradish peroxidase and ECL.

Expression of MT1-MMP and Treatment with MMP Inhibitors—To express full-length human MT1-MMP, confluent cultures of BS-C-1 or *Timp2* (−/−) mutant cells in six-well plates were co-infected with 5–10 plaque-forming units/cell each of vTF7–3 and vT7-MT1 viruses for 45 min in infection medium (DMEM + 2.5% fetal bovine serum and antibiotics) at 37 °C. As a control, the cells were infected only with the vTF7–3 virus. After infection, the cultures were incubated (12 h, 37 °C) with serum-free DMEM supplemented with various protease inhibitors: TIMP-2, TIMP-4, and TIMP-1 (0–100 nM); marimastat (0–500 nM); SB-3CT (0–100 nM); aprotinin (20 and 40 μg/ml); leupeptin (40 μg/ml); or E64 (10 μM) overnight, as described (20). TPA-treated HT1080 cells, as described above, received 100 μg/ml aprotinin or 100 nM human recombinant TIMP-2. The serum-free conditioned media were collected and processed for immunoblot analysis, as described below.

Cloning, Expression, and Isolation of Recombinant MT1-MMP Mutants—A catalytically inactive mutant of MT1-MMP was generated by replacing Glu^{240} with Ala (E240A-MT1) using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). A cytosolic domain (CD) deletion mutant (ΔCD-MT1) was constructed by introducing a termination codon at Arg^{563} by polymerase chain reaction (PCR) using specific primers and wild-type MT1-MMP cDNA as the template. The amplified E240A-MT1 and ΔCD-MT1 cDNA fragments were cloned into the pTF7EMCV-1 vaccinia expression vector using appropriate restriction sites to generate the respective expression vectors pTF7-E240A-MT1 and pTF7-ΔCD-MT1, as described (28, 33). The sequence of the inserts was verified by DNA sequencing. Expression of the MT1-MMP mutants was carried out in BS-C-1 cells by the infection/transfection procedure (28, 32, 33). Briefly, BS-C-1 cells were grown in 100-mm culture dishes to 80% confluence and infected with 5 plaque-forming units/cell vTF7–3 virus in infection medium for 45 min, as described (32). The cells were washed with phosphate-buffered saline and then transfected with 2 μg/dish pTF7-MT1 (wild type MT1-MMP), pTF7-E240A-MT1, or pTF7-ΔCD-MT1 DNA plasmids using Effectene transfection reagent (Qiagen, Valencia, CA), as described by the manufacturer. Control cells were infected but received no plasmid DNA. Four h after transfection, the cells were metabolically labeled (12 h, 37 °C) with 100 μCi/ml [^{35}S]methionine. The media were collected, clarified by centrifugation, and concentrated to ~0.5 ml followed by immunoprecipitation, as described below.

To express the 21-kDa (Tyr^{112}–Gly^{284}) and 18-kDa (Tyr^{112}–Ala^{255}) fragments of MT1-MMP, we utilized high fidelity PCR to amplify the respective cDNA fragments, which were cloned into the *Nde*I and *Hin*dIII restriction sites of the pET-24a(+) expression vector (Novagen, Madison, WI). The recombinant plasmid vectors were introduced into recipient *Escherichia coli* BL21(DE3) by transformation. For protein expression, 5 ml of the bacterial cultures were induced overnight at 37 °C with 0.4 mM isopropyl-β-D-galactopyranoside. Cells were pelleted by centrifugation, resuspended in 0.5 ml of collagenase buffer, and sonicated. Inclusion bodies were collected by centrifugation and dissolved in collagenase buffer containing 8 M urea. The solubilized proteins were diluted 10-fold in collagenase buffer supplemented with 50% glycerol and dialyzed overnight against collagenase buffer with 10% glycerol. The MT1-MMP fragments were resolved by 15% SDS-PAGE and stained with Coomassie Blue. The same samples were also analyzed for enzymatic activity and ability to bind TIMP-2 as described below.

Immunoaffinity Purification and Microsequencing of the 18-kDa MT1-MMP Fragment—BS-C-1 cells in 150-mm tissue culture dishes were infected to express MT1-MMP as described (17). Three h after infection, the cells were rinsed with serum-free media and incubated overnight with 15 ml/dish serum-free DMEM. The media (~300 ml) were collected, clarified by centrifugation, and concentrated ~15–20-fold with Centricon Plus-80. The concentrated medium was incubated (12 h, 4 °C) with pAb 160/Protein A-agarose beads to immunoprecipitate the MT1-MMP forms. The beads were washed twice with serum-free DMEM and twice with HNTG buffer (50 mM Tris/HCl, pH 7.5,

150 mM NaCl, 0.1% Nonidet P-40, and 10% glycerol). The immunoprecipitated proteins were eluted with reducing sample buffer, boiled and subjected to 15% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Coomassie Blue R-250. As reference, an aliquot of the eluate was also subjected to immunoblot analysis using pAb 160. The Coomassie Blue-stained 18-kDa protein was cut out from the polyvinylidene difluoride membrane and was submitted for N-terminal microsequencing by Edman-based chemistry to ProSeq (Boxford, MA). The amino acid sequence of the 18-kDa protein was also determined by mass spectrometry. To this end, the 18-kDa form bound to pAb 160/Protein A-agarose beads was eluted with 100 mM glycine/HCl, pH 2.5, and the collected fractions were neutralized with 1.88 M Tris, pH 8.8. An aliquot of each fraction was subjected to immunoblot analysis using pAb 160 to identify the 18-kDa protein. The fractions with the 18-kDa fragment were concentrated and subjected to 4–20% SDS-PAGE followed by Coomassie Blue R-250 staining. The protein was cut out from the gel and sent to the Harvard Microchemistry Facility for sequence analysis by microcapillary reverse-phase high performance liquid chromatography nano-electrospray tandem mass spectrometry on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer.

Computational Modeling—A full computational model (minus the hinge region) for the ectodomain of pro-MT1-MMP was developed for the studies of the various aspects of the biochemistry of this enzyme. The primary sequence of MT1-MMP was obtained from Swiss-Prot data bank (code MM14_HUMAN). An initial model of MT1-MMP was generated by homology modeling with the aid of COMPOSER software, implemented in the SYBYL package version 6.7, and it was further refined with energy minimization procedures. The catalytic and propeptide domains were constructed based on the structure of stromelysin-1 (Protein Data Bank identification code 1slm) (41) following similar procedures that were used for construction of the hemopexin-like domain (42). Individual domains of MT1-MMP were thus constructed using homology modeling and three-dimensional structure alignment, except for the catalytic domain, which was based on the published x-ray structure of the catalytic domain of MT1-MMP (Protein Data Bank identification code 1bqq) (43). The hinge region of MT1-MMP was not modeled because of the lack of any homologous protein that could serve as a three-dimensional template. Energy minimization of the complete MT1-MMP complex was carried out using the SANDER module of the AMBER 5.0 suite of programs (44). The force field of Cornell *et al.* (45) was used to model the enzyme. The parameters for bonds, angles, and van der Waals interactions involving zinc atoms were taken from Massova *et al.* (46). The enzyme was immersed in a $97 \times 96 \times 98$-Å^3 box of TIP3P-water (47). Water molecules present in the x-ray crystallographic structure were retained in the model. A 10-Å cutoff was applied to the model, and the nonbonded list was updated every 50 cycles. A total of 20,000 energy minimization cycles were carried out, which consisted of 300 steepest descent steps, followed by conjugate gradient minimization.

Enzyme Kinetic and Inhibition Studies—All enzymatic assays were carried out using the fluorescence substrate MOCAcPLGLA$_2$pr(Dnp)A-RNH$_2$ (Peptides International, Louisville, KY) in a buffer consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Brij-35, and 1% Me_2SO (buffer R). Substrate hydrolysis was monitored with a Photon Technology International spectrofluorometer at excitation and emission wavelengths of 328 and 393 nm, respectively. The synthetic peptide MOCAcPLG (Peptides International) was used to calibrate the assays as described by Knight (48). Concentrations of MT1-MMP$_{cat}$ (Tyr^{112}–Gly^{288}) and that of the recombinant 21-kDa (Tyr^{112}–Gly^{284}) fragment were determined by titration with TIMP-2. The TIMP-2 concentration was determined using a molar extinction coefficient of 39,600 M^{-1} cm^{-1} (49). Concentration of the 18-kDa (Tyr^{112}–Ala^{255}) fragment was determined by the BCA protein assay (Pierce) relative to a calibration curve established with the recombinant 21-kDa (Tyr^{112}–Gly^{284}) fragment as a standard. The kinetic parameters k_{cat} and K_m for the reaction of the recombinant 18-kDa (Tyr^{112}–Ala^{255}) and 21-kDa (Tyr^{112}–Gly^{284}) MT1-MMP species with the fluorogenic substrate were determined by computer fitting the substrate concentration dependence of the initial rates of substrate hydrolysis to the Michaelis-Menten equation using the program Scientist (MicroMath, Salt Lake City, UT). The fluorogenic substrate concentration was varied between 0.1 and 11 μM, where the extent of trivial quenching of the substrate is insignificant. Inhibition studies were carried out as previously described (20). The determination of k_{off} was attempted from the enzyme activity recovered after dilution of a pre-formed enzyme-inhibitor complex. The dissociation of the MT1-MMP·TIMP-2 complex, however, was too slow for the direct analysis of the k_{off} parameter for TIMP-2. The k_{off} value was estimated based on a 10-fold difference observed between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMP$_{cat}$ with a C-terminal deletion TIMP-2 mutant (ΔCTD-TIMP-2) (steady state rate) and wild type TIMP-2, as previously described (20).

MT1-MMP-TIMP-2 Interactions—Binding of soluble MT1-MMP forms to TIMP-2 was examined using various approaches. (i) Serum-free ^{35}S-labeled media (1 ml) from BS-C-1 cells expressing or not MT1-MMP were incubated (4 h, 4 °C) with or without 100 ng of either recombinant TIMP-2 or TIMP-1. The samples that received TIMP-2 or TIMP-1 were immunoprecipitated with anti-TIMP-2 or anti-TIMP-1 antibodies and Protein G-agarose beads. The samples without TIMP addition were immunoprecipitated with anti-MT1-MMP antibodies and Protein A-agarose beads. The immunoprecipitates were resolved by reducing 15% SDS-PAGE followed by autoradiography. (ii) Conditioned medium of BS-C-1 cells infected to express MT1-MMP in the presence of 1 μM marimastat, to induce the appearance of the 31–35-kDa species, was subjected to TIMP-2-affinity binding using immobilized TIMP-2 on an Affi-Gel 10 matrix, prepared as previously described (17). Briefly, the medium was concentrated (~80-fold), and ~0.4 ml of the concentrated medium (0.3 μM final marimastat concentration) was incubated (12 h, 4 °C) with Affi-Gel 10-TIMP-2 matrix by continuous rotation. After a brief centrifugation, the supernatant containing the unbound proteins was collected. The bound proteins were eluted with reducing Laemmli sample buffer (40). The bound and unbound fractions were resolved by reducing 15% SDS-PAGE followed by immunoblot analysis. (iii) Purified recombinant 18- and 21-kDa MT1-MMP fragments were incubated (1 h, 4 °C) with TIMP-2 (1:1 molar ratio) in 50 μl of collagenase buffer followed by immunoprecipitation with anti-TIMP-2 or anti-MT1-MMP antibodies. The complexes were detected by immunoblot analysis.

Immunoprecipitation—For immunoprecipitation of soluble MT1-MMP forms, serum-free media from ^{35}S-labeled cells expressing recombinant or natural MT1-MMP were immunoprecipitated with pAb 160/Protein A-agarose beads under nondenaturing conditions as described (15). In some experiments, the ^{35}S-labeled media were concentrated (10-fold) before immunoprecipitation. To immunoprecipitate denatured samples, the concentrated media were supplemented with 10× harvest buffer and boiled. The samples then received 2.5% Triton X-100 (final concentration) followed by addition of either pAb 437 or pAb 160 and Protein A-agarose beads as previously described (17). MT1-MMP·TIMP-2 complexes in ^{35}S-labeled medium samples or in mixtures of recombinant MT1-MMP fragments and TIMP-2 were co-immunoprecipitated with mAb 101 to TIMP-2 under nondenaturing conditions as previously described (33). The immunoprecipitates were resolved by reducing 15% SDS-PAGE, followed by autoradiography or by immunoblot analysis.

Gelatin Zymography and Immunoblot Analysis—Gelatin zymography was performed using 10% or 15% Tris-glycine SDS-polyacrylamide gels containing 0.1% gelatin as described (38). For immunoblot analysis, the serum-free conditioned media were collected, clarified by centrifugation, and concentrated (~80-fold) on a Centricon Plus-20 concentrator (Fisher, Itasca, IL). An aliquot was resolved by reducing 15% SDS-PAGE followed by transfer to nitrocellulose membrane. The membranes were incubated with the appropriate antibodies as described (38). The immunocomplexes were detected by ECL according to the manufacturer's instructions (Pierce).

Pro-MMP-2 Activation—Purified human pro-MMP-2 (25 nM) in collagenase buffer was incubated (22 h, 37 °C) with either 5 nM MT1-MMP$_{cat}$ (Calbiochem) or recombinant 18- and 21-kDa MT1-MMP fragments or concentrated serum-free conditioned media from BS-C-1 cell expressing MT1-MMP or from BS-C-1 cells infected only with the T7 RNA polymerase-expressing virus (vTF7–3). As a positive control, pro-MMP-2 was activated with 1 mM *p*-aminophenylmercuric acetate for 1 h at 37 °C. Pro-MMP-2 activation was monitored by gelatin zymography

RESULTS

Membrane-bound and Soluble Forms of MT1-MMP—We have previously shown that active MT1-MMP (57 kDa) is autocatalytically processed to a major membrane-bound 44-kDa species starting at Gly^{285} (17). This processing should release the entire catalytic domain of MT1-MMP. We therefore examined the surface and extracellular distribution of MT1-MMP in various cell lines known to express natural MT1-MMP and in cells engineered to express recombinant MT1-MMP as a model system. In cells expressing natural MT1-MMP, these studies

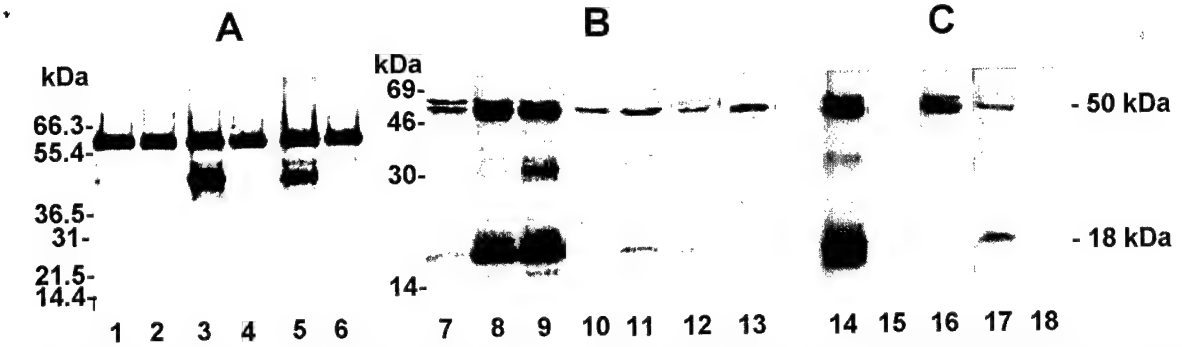

FIG. 1. **Membrane-bound and soluble MT1-MMP forms.** *A*, biotinylation of membrane-bound MT1-MMP forms. HT1080 cells were untreated (*lanes 1* and *2*) or treated with either 100 nM TPA (*lanes 3* and *4*) or 10 μg/ml ConA (*lanes 5* and *6*) in serum-free media overnight. The cells were then surface-biotinylated, as described (38). The biotinylated MT1-MMP forms were immunoprecipitated with pAb 437 (*lanes 1*, *3*, and *5*) or with pAb 160 (*lanes 2*, *4*, and *6*). The immunoprecipitates were resolved by reducing 8–16% SDS-PAGE, transferred to a nitrocellulose membrane, and detected with streptavidin-horseradish peroxidase and ECL. *B*, immunoblot analysis of soluble MT1-MMP forms. HT1080 (*lanes 7–9*), HFL1 (*lanes 10* and *11*), U-87 (*lane 12*), and MDA-MB-231 (*lane 13*) cells were untreated (*lanes 7* and *10*) or treated with either 100 nM TPA (*lane 8*) or 10 μg/ml ConA (*lanes 9*, *11*, *12*, and *13*) in serum-free media overnight. The concentrated (~80-fold) conditioned media were analyzed by immunoblot analysis using the anti-MT1-MMP mAb LEM-2/15. *C*, immunoprecipitation of soluble MT1-MMP forms. HT1080 (*lanes 14* and *15*), MDA-MB-231 (*lane 16*) and *Timp2* (−/−)-MT1 (*lanes 17* and *18*) cells were metabolically labeled with [^{35}S]methionine as described under "Experimental Procedures." HT1080 and MDA-MB-231 cells were treated with 100 nM TPA or 10 μg/ml ConA, respectively, in serum-free media during metabolic labeling. The ^{35}S-labeled media were immunoprecipitated with pAb 160/Protein A-agarose beads (*lanes 14*, *16*, and *17*) or with Protein A-agarose beads without antibody (*lanes 15* and *18*). The immunoprecipitates were resolved by reducing 15% SDS-PAGE followed by autoradiography.

were carried out either without stimulation or after stimulation with TPA or ConA, two agents known to induce MT1-MMP expression (10, 16, 29, 50). Surface biotinylation followed by immunoprecipitation with pAb 160 to the catalytic domain or pAb 437 to the hemopexin-like domain demonstrated that untreated HT1080 cells display the 57-kDa species of MT1-MMP as the major enzyme form on the cell surface (Fig. 1*A*, *lanes 1* and *2*). Treatment with either TPA (Fig. 1*A*, *lanes 3* and *4*) or ConA (Fig. 1*A*, *lanes 5* and *6*) induced the appearance of a 44-kDa species on the cell surface, which was detected only with the pAb 437 (Fig. 1*A*, *lanes 3* and *5*), indicating that this species represents a membrane-inserted form lacking the catalytic domain, in agreement with our previous studies using a vaccinia expression system (17). Thus, both TPA and ConA promote the processing of natural MT1-MMP (57 kDa) into the inactive 44-kDa form.

We next examined the serum-free conditioned media of various cell lines (untreated or treated with TPA or ConA) and *Timp-2* (−/−) mouse fibroblasts stable transfected to express recombinant MT1-MMP for soluble MT1-MMP forms by immunoblot analysis (Fig. 1*B*) and immunoprecipitation (Fig. 1*C*). As shown in Fig. 1*B*, the medium of untreated HT1080 cells contains three proteins of 56, 50, and 18 kDa, which were recognized by a mAb to the catalytic domain of MT1-MMP (Fig. 1*B*, *lane 7*). TPA (Fig. 1*B*, *lane 8*) and ConA (Fig. 1*B*, *lane 9*) treatment of HT1080 cells enhanced the levels of these forms in the media and resulted in the appearance of an additional soluble MT1-MMP form of ~31–35 kDa, which was particularly evident with ConA (Fig. 1*B*, *lane 9*). Media of untreated HFL1 fibroblasts (Fig. 1*B*, *lane 10*) and U-87 glioblastoma cells (data not shown) showed presence of the 50-kDa species. Media of ConA-treated HFL1 (Fig. 1*B*, *lane 11*) and U-87 (Fig. 1*B*, *lane 12*) cells contained the 50- and 18-kDa species and very low levels of the ~31–35-kDa species. Media of ConA-treated MDA-MB-231 contained mostly the 50-kDa form, as determined by immunoblot analysis (Fig. 1*B*, *lane 13*) or immunoprecipitation (Fig. 1*C*, *lane 16*). With the exception of the 18- and the 31–35-kDa species, the 56- and 50-kDa species were recognized by pAb 437 to the hemopexin-like domain, indicating that they comprise most of the ectodomain (data not shown).

Metabolic labeling of TPA-treated HT1080 cells followed by immunoprecipitation with pAb 160 yielded the 56-, 50-, 31–35-, and 18-kDa species (Fig. 1*C*, *lane 14*), whereas the same procedure in *Timp-2* (−/−) cells expressing MT1-MMP yielded mostly the 50- and 18-kDa species (Fig. 1*C*, *lane 17*). No signal was observed in samples precipitated with Protein A-agarose beads without antibody (Fig. 1*C*, *lanes 15* and *18*). Considering that the 18-kDa species contains only one methionine residue (based on sequencing data, as shown below), the results of the immunoprecipitation of the ^{35}S-labeled media indicate that the 18-kDa species, compared with the other forms, exhibits a relatively higher specific activity and hence represents the major soluble form of MT1-MMP.

The concentrated serum-free media of untreated HT1080 cells and BS-C-1 cells expressing MT1-MMP were subjected to ultracentrifugation (100,000 × *g* for 1 h at 4 °C) to assess the distribution of the released MT1-MMP species. Under these conditions, membrane fragments and their associated proteins and large protein aggregates go to the pellet, whereas membrane-free soluble species distribute mostly in the supernatant (51, 52). Immunoblot analysis showed that the 18-, 31–35-, and 50-kDa species were detected in the supernatant, whereas the 56-kDa species remained in the pellet (data not shown). These results suggest that, with the exception of the 56-kDa species, the other MT1-MMP species are true soluble forms.

Complex Regulation of MT1-MMP Shedding—We next investigated the effects of various protease inhibitors on the profile of MT1-MMP forms present in the media. To this end, we used TPA-treated HT1080 cells (Fig. 2*A*), which express natural MT1-MMP and the *Timp2* (−/−) cells (Fig. 2*C*) expressing recombinant MT1-MMP. In addition, we used BS-C-1 cells infected with vaccinia virus expressing MT1-MMP, as we have previously reported (17, 20). The profile of soluble MT1-MMP species found in the vaccinia expression system (shown in Fig. 2*B*) was the same as that observed in cells expressing natural MT1-MMP (Fig. 1) and hence is not a consequence of overexpression of recombinant enzyme or cell lysis. Thus, this experimental system recapitulates the natural pattern of MT1-MMP shedding. The protease inhibitor studies showed that presence of the 18-kDa species in the media was specifically inhibited by TIMP-2, marimastat, and TIMP-4 in all cells tested (Fig. 2, *A–C*). TIMP-1, an extremely poor MT1-MMP inhibitor (53) (Fig. 2*B*, *lane 7*), and SB-3CT, a mechanism-based synthetic inhibitor specific for the gelatinases (36) (Fig.

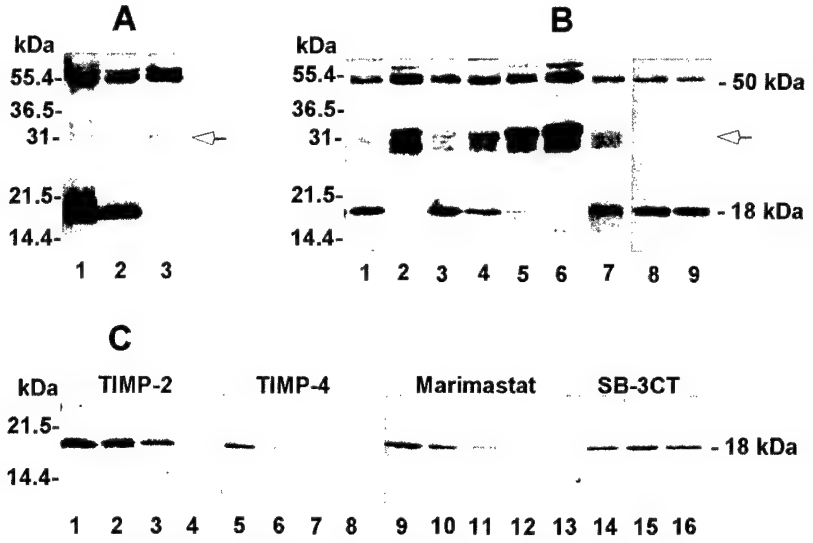

FIG. 2. **Effect of protease inhibitors in MT1-MMP shedding.** *A*, HT1080 cells were treated with 100 nM TPA overnight in serum-free media in the absence (*lane 1*) or presence of 100 μg/ml aprotinin (*lane 2*) or 100 nM human recombinant TIMP-2 (*lane 3*). The conditioned media were collected, concentrated (~80-fold), and subjected to reducing 15% SDS-PAGE followed by immunoblot analysis using the anti-MT1-MMP mAb (LEM-2/15). *B* and *C*, BS-C-1 (*B*) or *Timp-2* (−/−) (*C*) mutant cells were co-infected with vaccinia viruses to express MT1-MMP as described under "Experimental Procedures." After infection, the media were aspirated and replaced with serum-free DMEM supplemented without or with various concentrations of protease inhibitors for overnight incubation. The conditioned media were collected and subjected to reducing 15% SDS-PAGE followed by immunoblot analysis using pAb 160. Inhibitor doses in *B* were as follows: none (*lane 1*); TIMP-2, 20 nM (*lane 2*); marimastat, 10^{-6}, 10^{-4}, 10^{-2}, and 1 μM (*lanes 3*, *4*, *5*, and *6*, respectively); TIMP-1, 100 nM (*lane 7*); and aprotinin, 20 and 40 μg/ml (*lanes 8* and *9*, respectively). Inhibitor doses in *C* were as follows: TIMP-2, 0, 1, 10, and 100 nM (*lanes 1*, *2*, *3*, and *4*, respectively); TIMP-4, 0, 1, 10, and 100 nM (*lanes 5*, *6*, *7*, and *8*, respectively); marimastat, 0, 4, 20, 100, and 500 nM (*lanes 9*, *10*, *11*, *12*, and *13*, respectively); and SB-3CT, 4, 20, and 100 nM (*lanes 14*, *15*, and *16*, respectively). *Open arrows* show the 31–35-kDa fragment.

2*C*, *lanes 14–16*), had no effect. Aprotinin (Fig. 2, *A* (*lane 2*) and *B* (*lanes 8* and *9*)) and leupeptin (40 μg/ml; data not shown), two serine protease inhibitors, and E64 (10 μM), an aspartic protease inhibitor (data not shown), had no effect on the shedding of the 18-kDa species. None of the inhibitors tested had a significant effect on the levels of the 50-kDa form and in fact, the levels of this species were somewhat increased in the presence of TIMP-2 and marimastat in BS-C-1 cells (Fig. 2*B*, *lanes 2* and *6*, respectively) but not in HT1080 cells (Fig. 2*A*, *lane 3*). Interestingly, we also found that, both in HT1080 cells (Fig. 2A, *lane 3*) and in BS-C-1 cells expressing MT1-MMP (Fig. 2B, *lanes 2–6*), the ~31–35-kDa species accumulated in the presence of TIMP-2 and marimastat but not in the presence of aprotinin (Fig. 2, *A* (*lane 2*) and *B* (*lanes 8* and *9*)).

The inhibitor profile studies suggested that shedding of the 18-kDa species is an autocatalytic event, whereas shedding of the 50-kDa species is not. To further investigate this process, we generated a catalytically inactive mutant of MT1-MMP by replacing Glu^{240} with Ala (E240A-MT1). We also examined the role of the cytosolic domain of MT1-MMP in shedding. To this end, we constructed a truncated MT1-MMP lacking the cytosolic domain (ΔCD-MT1) by introducing a stop codon at Arg^{563}. Wild type MT1-MMP and the E240A-MT1 and ΔCD-MT1 mutants were expressed in BS-C-1 cells using the infection-transfection procedure followed by metabolic labeling as described under "Experimental Procedures." The ^{35}S-labeled conditioned media were immunoprecipitated with pAb 160. As shown in Fig. 3, wild type MT1-MMP shed the 50-, 31–35-, and 18-kDa species (Fig. 3, *lane 1*). In contrast, the E240A-MT1 catalytic mutant shed the 50-kDa species and a ~28-kDa form but not the 18-kDa fragment (Fig. 3, *lane 2*) consistent with the autocatalytic shedding of the latter species. The ΔCD-MT1 mutant showed a shedding pattern similar to that observed with the wild type enzyme (Fig. 3, *lane 3*).

Structure and Characterization of the 18-kDa MT1-MMP Soluble Form—The relatively higher amounts of the 18-kDa fragment allowed its purification and characterization. N-ter-

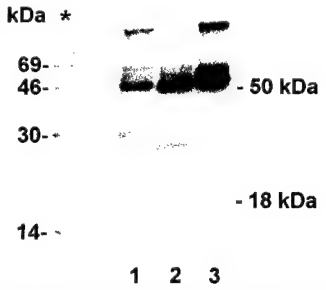

FIG. 3. **Shedding in MT1-MMP mutants.** Wild type human MT1-MMP (*lane 1*), E240A-MT1 (*lane 2*), and ΔCD-MT1 (*lane 3*) MT1-MMP mutants were expressed in BS-C-1 cells by infection/transfection as described under "Experimental Procedures" followed by metabolic labeling (12 h, 37 °C) with [^{35}S]methionine. The ^{35}S-labeled medium was subjected to immunoprecipitation with pAb 160/Protein A-agarose beads, and the immunoprecipitates were resolved by reducing 15% SDS-PAGE followed by autoradiography. The *asterisk* indicates the lane with ^{14}C-labeled molecular size standards.

minal sequencing by Edman degradation revealed that the 18-kDa species displays Tyr^{112} in the N terminus, in agreement with the N terminus displayed by membrane-tethered active MT1-MMP (57 kDa) (9, 17). Mass spectrometry analysis of tryptic digests was performed to determine the C terminus of the 18-kDa fragment. As shown in the table of Fig. 4*A* (*inset*), a total of 32 peptides were isolated and their sequence determined. Three peptides demonstrated a C terminus ending at Ala^{255}, indicating that the 18-kDa form extends from Tyr^{112} to Ala^{255} and therefore comprises most of the catalytic domain. Indeed, SDS-PAGE analysis demonstrated an ~3-kDa difference between the shed 18-kDa fragment (Fig. 4*B*, *lane 2*) and a commercially available recombinant catalytic domain of MT1-MMP ($MT1\text{-}MMP_{cat}$), which is known to extend from Tyr^{112} to Gly^{288} (Fig. 4*B*, *lane 1*).

Considering that the 44-kDa membrane-tethered species of MT1-MMP starts at Gly^{285} (17), shedding of the 18-kDa fragment would require two cleavage events: one between Ala^{255}

A

Number of Peptides and Sequences	AA# in MT1-MMP
2 YAIQGLK	112-118
1 WQHNEITFCIQNYTPK	119-134
2 VGEYATYEAIR	135-145
2 VGEYATYEAIRK	135-146
2 VWESATPLR	150-158
2 ESATPLR	152-158
3 EVPYAYIR	161-168
1 PYAYIR	163-168
2 QADIMIF	174-180
4 AYFPGPNIGGDTHFDSAEPWTVR	202-224
1 PGPNIGGDTHFDSAEPWTVR	205-224
4 IGGDTHFDSAEPWTVR	209-224
1 AYFPGPN	202-208
2 NEDLNGNDIFLVAVHELGH	225-243
1 VHELGHALGLEHSSDPSA	238-255
2 HALGLEHSSDPSA	244-255

B

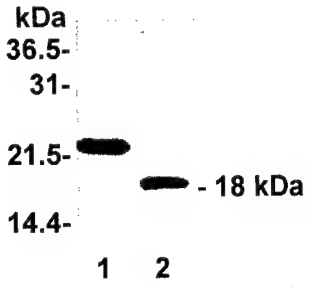

FIG. 4. **Characterization of the 18-kDa MT1-MMP soluble fragment.** *A*, summary of the tandem mass spectrometry peptide sequences of the 18-kDa form. The last two peptides in the *highlighted box* comprise the C-terminal region of the 18-kDa fragment. *AA*, amino acid. *B*, immunoblot analysis of 20 ng of recombinant MT1-MMP$_{cat}$ (Tyr^{112}–Gly^{288}) (*lane 1*) and concentrated serum-free medium of *Timp-2* (−/−) mutant cells infected to express MT1-MMP. The antigens were detected with pAb 160 and ECL.

and Ile^{256} and another between Gly^{284} and Gly^{285}. To determine the relative location of these sites in the catalytic domain of MT1-MMP, we used an energy-minimized computational model of the ectodomain of human pro-MT1-MMP that was recently constructed in our laboratory.[2] The only missing piece of structural information in this model pertained to the hinge between the catalytic and the hemopexin-like domains. The spatial *location of the hemopexin-like domain in the computational model (data not shown) was based on that seen in the x-ray structure of MMP-2 (54). From this model, Fig. 5A* shows only a view of the catalytic domain extending from Tyr^{112} to Ser^{287}. In the event that the hinge actually would not dislocate the hemopexin-like domain in MT1-MMP, the Ala^{255}-Ile^{256} bond is sheltered by the hemopexin-like domain, leaving the cleavage site Gly^{284}-Gly^{285} as the only likely candidate for the first hydrolytic cleavage event. However, even if the hemopexin-like domain of MT1-MMP is dislocated away from the catalytic domain, leaving the surface regions shown in Fig. 5*B* fully exposed to the milieu, still the cleavage site Gly^{284}-Gly^{285} is more readily accessible than is Ala^{255}-Ile^{256} because of the nature of the secondary structures in the protein. Therefore, shedding of the 18-kDa fragment is likely to be initiated at the Gly^{284}-Gly^{285} peptide bond, followed by a second cleavage at the Ala^{255}-Ile^{256} site. Fig. 5*C* depicts a diagram of active MT1-MMP (Tyr^{112}–Val^{582}), showing the two cleavages at the Gly^{284}-Gly^{285} and Ala^{255}-Ile^{256} sites leading to the formation of the inactive 44-kDa species, which has been isolated and characterized from plasma membranes (17), and the soluble 18-kDa species. To shed the 18-kDa species, this process would have also generated a ~21-kDa intermediate fragment (Fig. 5*C*, *dashed bracket*) extending from Tyr^{112} to Gly^{284}. However, a soluble fragment of ~21 kDa, the putative precursor of the 18-kDa species, was not detected. Additionally, hydrolysis at the Ala^{255}-Ile^{256} peptide bond predicted impaired catalytic activity of the 18-kDa species because of the proximity of this site to the conserved methionine residue (Met^{257}) of the so-called methionine turn (55) and to the consensus sequence of the catalytic zinc ion binding site (Fig. 5*C*).

To gain insight into the biochemical properties of the 18-kDa (Tyr^{112}–Ala^{255}) fragment and the putative 21-kDa (Tyr^{112}–Gly^{284}) intermediate species, these proteins were expressed in bacteria and purified to homogeneity for further analyses. Fig. 6*A* shows the purity of the recombinant 18-kDa (Tyr^{112}–Ala^{255}) (*lane 1*) and 21-kDa (Tyr^{112}–Gly^{284}) (*lane 2*) proteins isolated. Activity assays demonstrated that, whereas the 21-kDa (Tyr^{112}–Gly^{284}) fragment exhibited gelatinolytic (Fig. 6*B*, *lane 4*) and pro-MMP-2-activating activities (Fig. 6*C*, *lane 6*), the 18-kDa (Tyr^{112}–Ala^{255}) fragment was catalytically inactive (Fig. 6, *B* (*lane 3*) and *C* (*lane 5*)). To obtain quantitative data, the recombinant fragments were examined for their ability to hydrolyze a fluorogenic peptide substrate as a function of time. MT1-MMP$_{cat}$ was used as a positive control. As summarized in Table I, k_{cat} and K_m values of ~1 s^{-1} and 10 μM, respectively, were obtained yielding k_{cat}/K_m values of 10^5 M^{-1} s^{-1}, which reflects the high reactivity of MT1-MMP$_{cat}$ and the 21-kDa (Tyr^{112}–Gly^{284}) enzymes toward the synthetic peptide substrate used. Moreover, indistinguishable values were obtained for these two MT1-MMP species. In contrast, no enzyme concentration dependence of the rate of substrate hydrolysis was detected with the 18-kDa (Tyr^{112}–Ala^{255}) fragment with concentrations up to 235 nM. In fact, the hydrolysis rate of the substrate in the presence of the enzyme was essentially indistinguishable from the background hydrolysis detected in buffer only. A comparable concentration of the 21-kDa (Tyr^{112}–Gly^{284}) species (54 nM) yielded an increase in fluorescence that rapidly exceeded the detection limit of the instrument used. Together, these studies indicate that autocatalytic processing of MT1-MMP at the Ala^{255}-Ile^{256} site obliterates catalytic competence resulting in an inactive soluble form of 18 kDa.

Although the 18-kDa species of MT1-MMP is inactive, the other soluble species may maintain enzymatic activity. Unfortunately, the paucity of these enzyme species in the media precluded purification and characterization. Thus, to investigate the activity of the soluble MT1-MMP species, we used conditioned medium of BS-C-1 cells infected to express MT1-MMP and examined its ability to promote pro-MMP-2 activation after addition of exogenous recombinant pro-MMP-2. This cell expression system was chosen because it is devoid of MMP-2 (33) and because it releases the 50- and 18-kDa form of MT1-MMP into the media (Fig. 2*B*, *lane 1*). As a control, we used conditioned media of BS-C-1 cells infected only with the T7 RNA polymerase-expressing vaccinia vTF7–3 virus (33). As shown in Fig. 7, the conditioned media derived from the MT1-MMP-expressing cells promoted the generation of the intermediate form of MMP-2 (Fig. 7, *lane 6*), consistent with the two-step model of surface activation of pro-MMP-2 by MT1-MMP, as previously proposed (53, 56). In contrast, no processing of pro-MMP-2 was observed with the control media (Fig. 7, *lane 4*), demonstrating the specificity of the reaction. Because the 18-kDa species is inactive, these studies suggest that MT1-MMP fragments other than the 18-kDa form, such as the 50-kDa species, are likely to be the enzyme species responsible in the media for the processing of pro-MMP-2.

Interactions with TIMP-2—We have previously shown that, on the cell surface, TIMP-2 binds to the active 57-kDa form of MT1-MMP but not the 44-kDa inactive species (17), indicating that binding is mostly mediated by the catalytic domain. Here

[2] O. Meroueh, L. P. Kotra, R. Fridman, and S. Mobashery, unpublished data.

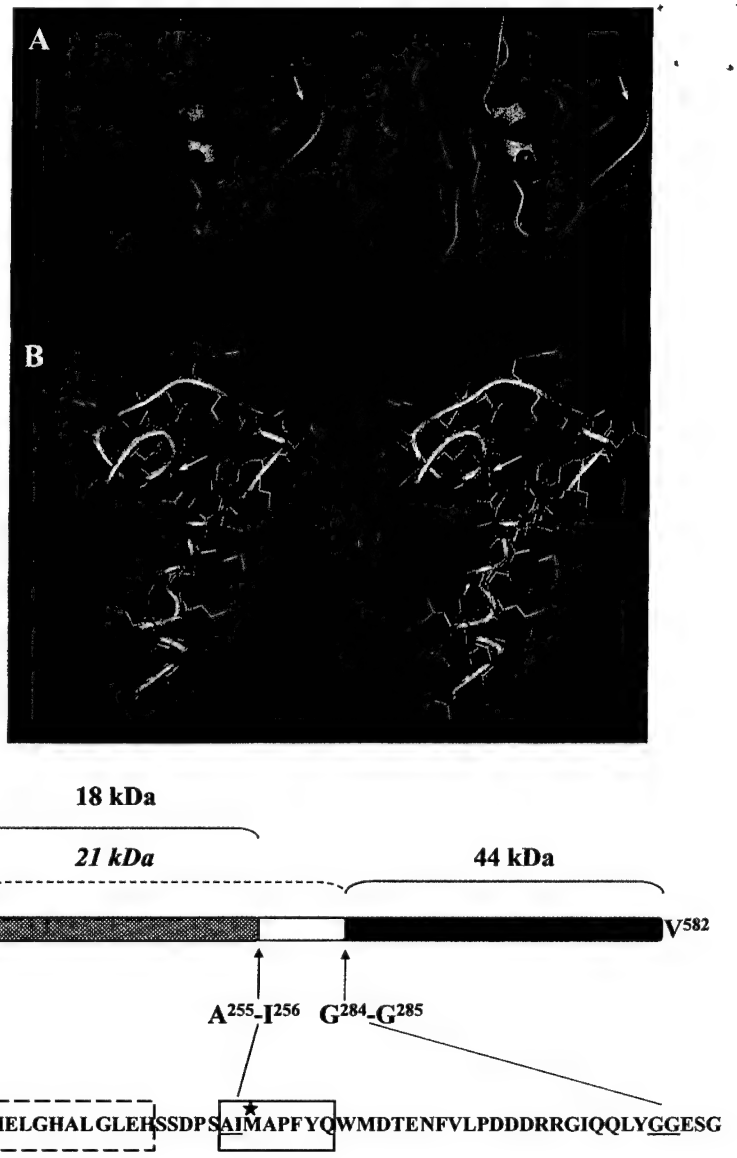

FIG. 5. **Computational model of MT1-MMP.** *A*, stereo view of the energy-minimized computational model of the MT1-MMP region spanning residues Tyr^{112} and Ser^{287}. *White* and *red arrows* are pointing to the Ala^{255}-Ile^{256} and Gly^{284}-Gly^{285} cleavage sites, respectively. *B*, a close-up view depicting the cleavage sites with *white* and *red arrows* corresponding to same cleavage sites described in *A*. Residues from the catalytic domain downstream of the Ala^{255}-Ile^{256} cleavage site are rendered in *white tube* representation, with the side chains shown. A Connolly water-accessible surface is constructed around the remaining residues. Residues at the cleavage sites are rendered in *yellow capped-sticks* representation. *B*, the Gly^{284}-Gly^{285} cleavage site is more readily accessible than that of Ala^{255}-Ile^{256}. *C*, diagram showing the location of the cleavage sites within active MT1-MMP (Tyr^{112}–Val^{582}) that lead to the formation of the membrane-inserted 44-kDa species (*black*) and the shedding of the 18-kDa species (*gray*). The *italics* and *dashed bracket* depict the putative 21-kDa intermediate species (*light gray*) predicted to precede the 18-kDa species. However, a 21-kDa species was not detected. Also shown is the sequence of amino acids around the cleavage sites including the conserved catalytic zinc-binding region (*dashed line rectangle*) and the conserved residues among transmembrane MT-MMPs (MT1-, MT2-, MT3-, and MT5-MMP) (*solid line rectangle*) containing the Ala^{255}-Ile^{256} site and the conserved methionine residue (marked by a *star*) comprising the methionine turn. The *underlines* indicate the Ala^{255}-Ile^{256} and Gly^{284}-Gly^{285} cleavage sites. The hinge region of MT1-MMP starts downstream of Gly^{285}.

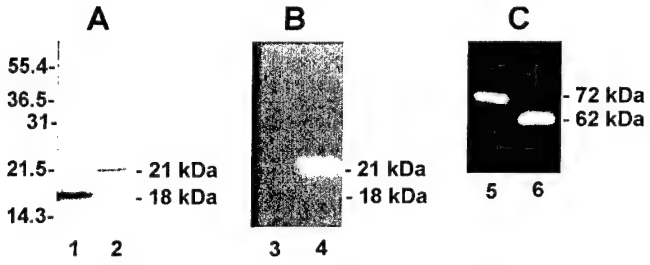

FIG. 6. **Activity of the recombinant 21- and 18-kDa MT1-MMP forms.** *A* and *B*, the purified recombinant 18-kDa (Tyr^{112}–Ala^{255}) (*lanes 1* and *3*) and 21-kDa (Tyr^{112}–Gly^{284}) (*lanes 2* and *4*) were subjected to reducing 15% SDS-PAGE followed by Coomassie Blue staining (*A*) and a 15% SDS-PAGE gelatin zymogram (*B*). *C*, pro-MMP-2 was incubated (12 h, 37 °C) with either the 18-kDa (Tyr^{112}–Ala^{255}) form (*lane 5*) or with the 21-kDa (Tyr^{112}–Gly^{284}) form (*lane 6*). An aliquot of the reaction was subjected to gelatin zymography. The 72-kDa form represents pro-MMP-2, and the 62-kDa form represents the fully active MMP-2.

TABLE I

Kinetic parameters for the reaction of MT1-MMP fragments with the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)ARNH$_2$

The fluorescence of reaction mixtures containing 0.2 nM enzyme and increasing concentrations of the fluorogenic substrate (0.1–11 μM) in buffer R was monitored at excitation and emission wavelengths of 328 and 393 nM, respectively. The kinetic parameters were determined by nonlinear least squares analysis of the substrate concentration dependence of initial hydrolysis rates as described under "Experimental Procedures."

Enzyme	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$M^{-1}\ s^{-1}$
MT1-MMP$_{cat}$ (Tyr^{112}–Gly^{288})	1.2 ± 0.3	10 ± 3	(1.25 ± 0.05) × 10^5
21-kDa (Tyr^{112}–Gly^{284})	3.3 ± 0.2	9 ± 1	(3.49 ± 0.05) × 10^5
18-kDa (Tyr^{112}–Ala^{255})	ND[a]	ND	ND

[a] ND, not detectable proteolytic activity of the enzyme toward the fluorogenic substrate was observed at concentrations up to 235 nM.

we examined the ability of the soluble MT1-MMP forms to bind TIMP-2. To this end, media of ^{35}S-labeled BS-C-1 cells infected to express MT1-MMP were incubated with or without exogenous recombinant TIMP-2 or TIMP-1 followed by immunoprecipitation. As shown in Fig. 8*A*, MT1-MMP-expressing BS-C-1 cells shed the 50- and 18-kDa species of MT1-MMP, as determined after immunoprecipitation with pAb 160 (Fig. 8*A*, *lane 1*). No signal was detected without antibody (Fig. 8*A*, *lane 2*). After addition of exogenous TIMP-2 and immunoprecipitation with mAb 101, only the 50-kDa species of MT1-MMP and some endogenously produced ^{35}S-TIMP-2 were detected in the coprecipitate (Fig. 8*A*, *lane 3*). Indeed, BS-C-1 cells produce very low levels of endogenous TIMP-2 (20). No signal was detected in samples that received exogenous TIMP-1 and anti-TIMP-1 pAb (Fig. 8*A*, *lane 4*). Accordingly, the 50-kDa species could not

FIG. 7. **Pro-MMP-2 activation by soluble MT1-MMP.** Recombinant pro-MMP-2 (25 nM) was incubated (37 °C) with either 5 nM MT1-MMP_{cat} (*lanes 1* and *2*) or with concentrated serum-free media from control infected BS-C-1 cells (*lanes 3* and *4*) or with concentrated media from BS-C1 cells expressing MT1-MMP (*lanes 5* and *6*). Samples were incubated for 0 min (*lanes 1, 3,* and *5*) and 22 h (*lanes 2, 4,* and *6*). Aliquots from the samples were subjected to gelatin zymography. *Lane 7* shows APMA-activated pro-MMP-2 (62 kDa) as a positive control.

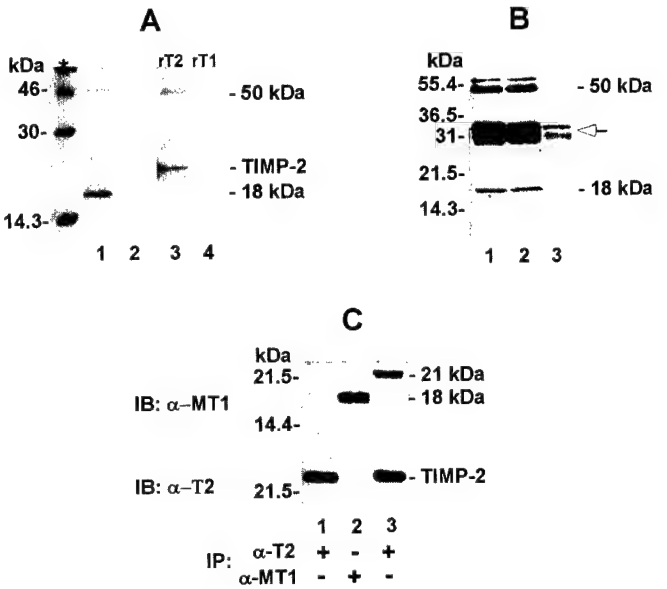

FIG. 8. **Interactions with TIMP-2.** *A*, ^{35}S-labeled-medium of BS-C-1 cells infected to express MT1-MMP was subjected to immunoprecipitation with pAb 160 and Protein A-agarose beads (*lane 1*) or Protein A-agarose beads alone (*lane 2*). An aliquot (1 ml) of the conditioned ^{35}S-labeled medium received 100 ng of unlabeled recombinant TIMP-2 (*rT2, lane 3*) or TIMP-1 (*rT1, lane 4*) followed by immunoprecipitation with anti-TIMP-2 (mAb 101) or anti-TIMP-1 pAb, respectively, and Protein G-agarose beads. The immunoprecipitates were resolved by reducing 15% SDS-PAGE followed by autoradiography. The *asterisk* indicates the lane with ^{14}C-labeled molecular size standards. *B*, BS-C-1 cells were co-infected with vaccinia viruses to express MT1-MMP. After infection the cells were incubated overnight in serum-free DMEM supplemented with 1 μM marimastat to induce appearance of the 31–35-kDa species. The conditioned media were collected, concentrated (~80-fold), and subjected to TIMP-2 affinity binding as described under "Experimental Procedures." The unbound and bound MT1-MMP forms were resolved by reducing 15% SDS-PAGE followed by immunoblot analysis using the mAb LEM-2/15. *Lane 1*, sample before affinity binding; *lane 2*, unbound fraction; *lane 3*, bound fraction. *C*, the recombinant 18-kDa (Tyr^{112}–Ala^{255}) (*lanes 1* and *2*) and 21-kDa (Tyr^{112}–Gly^{284}) (*lane 3*) MT1-MMP fragments were incubated (1 h) with TIMP-2 (1:1 molar ratio) in 50 μl of collagenase buffer. The samples were subjected to immunoprecipitation with pAb 160 to MT1-MMP (*lane 2*) or mAb 101 to TIMP-2 (*lanes 1* and *3*). The immunoprecipitates were resolved by 15% SDS-PAGE followed by immunoblot (*IB*) analysis with the same antibodies.

be detected with pAb 160 after TIMP-2 addition because of epitope occupancy in the enzyme-inhibitor complex as this pAb is directed to the catalytic domain (data not shown). These results indicate that TIMP-2, but not TIMP-1, binds to the soluble 50-kDa species via the catalytic domain, in agreement with the known TIMP-binding profile of MT1-MMP (53). In contrast, the soluble 18-kDa fragment cannot form a stable complex with TIMP-2.

A TIMP-2 affinity binding procedure was carried out to assess whether the 31–35-kDa species could bind TIMP-2. To this end, we used conditioned medium from BS-C-1 cells that were infected to express MT1-MMP in the presence of 1 μM marimastat, which induces the appearance of these species, as shown in Fig. 2*B*. The media were subjected to TIMP-2 affinity binding using immobilized TIMP-2, and the bound and unbound fractions were analyzed by immunoblot analysis as described under "Experimental Procedures." As shown in Fig. 8*B*, the 31–35- and the 50-kDa species were detected in the bound fraction (Fig. 8*B*, *lane 3*), albeit a significant amount of these species remained in the unbound fraction (Fig. 8*B*, *lane 2*) when compared with the load (Fig. 8*B*, *lane 1*), suggesting that they exhibit a relatively low affinity for TIMP-2, under the experimental conditions. In contrast, the 18-kDa species did not bind to the TIMP-2 affinity matrix, in agreement with the immunoprecipitation data. Although these studies are not quantitative, the poor binding of the soluble MT1-MMP species to the immobilized TIMP-2 is unlikely to be the result of the presence of marimastat because the affinity matrix is saturated with TIMP-2 and the final concentration of marimastat was less than 0.3 μM.

We next examined the ability of the recombinant MT1-MMP fragments to bind TIMP-2 by co-immunoprecipitation. These studies demonstrated that only the 21-kDa (Tyr^{112}–Gly^{284}) species was able to form a stable complex with the inhibitor (Fig. 8*C*, *lane 3*). Thus, loss of the 29 amino acids between Ala^{255} and Gly^{284} during the formation of the 18-kDa fragment strongly compromised TIMP-2 interactions. Consistently, inhibition studies demonstrated that TIMP-2 was an effective slow-binding inhibitor of the 21-kDa (Tyr^{112}–Gly^{284}) species when compared with MT1-MMP_{cat} (Table II). Fitting the data to a slow-binding inhibition model yielded k_{on} and k_{off} values of 10^6 M^{-1} s^{-1} and 2×10^{-4} s^{-1}, respectively, resulting in sub-nanomolar K_i values.

TABLE II
Kinetic parameters for inhibition of the 21-kDa MT1-MMP fragment by TIMP-2

The enzyme (0.2–0.8 nM) was added to a solution of $MOCAcPLGLA_2pr(Dnp)ARNH_2$ (10 μM) and increasing concentrations of TIMP-2 in buffer R. Substrate hydrolysis was monitored for 20 min. The kinetic parameters were evaluated as described under "Experimental Procedures."

Enzyme	k_{on}	k_{off}	K_i
	M^{-1} s^{-1}	s^{-1}	nM
MT1-MMP_{cat} (Tyr^{112}–Gly^{288})	$(3.54 \pm 0.56) \times 10^6$	2×10^{-4} [a]	0.06
21-kDa (Tyr^{112}–Gly^{284})	$(3.81 \pm 0.23) \times 10^6$	2×10^{-4} [a]	0.05

[a] Estimated value based on a 10-fold difference between the slopes of the linear portions of the dissociation curves for the complexes of MT1-MMP_{cat} with ΔCTD-TIMP-2 (steady state rate) and wild type TIMP-2.

DISCUSSION

The stability of active MT1-MMP on the cell surface is a complex process involving a balance between autocatalytic processing (17) and enzyme internalization (57, 58). Both processes can regulate the amount of active enzyme available for pericellular proteolysis and appear to be regulated in part by the presence of TIMPs. The major pathway of active MT1-MMP processing on the cell surface is an autocatalytic event that generates a 44-kDa membrane-anchored fragment starting at Gly^{285} and thus lacks the entire catalytic domain (17). This process may switch the proteolytic machinery from the cell surface to the pericellular space if the released fragments are competent enzymes or may obliterate proteolysis on all fronts, if the soluble fragments are inactive and/or in a complex with TIMPs. To define the fate of the catalytic domain after processing, we set out to investigate the nature and properties of the MT1-MMP soluble fragments produced in various cellular systems. Here we have shown that media of cells expressing natural or recombinant MT1-MMP contain a complex profile of

MT1-MMP species including two major species of 50- and 18 kDa and a series of minor fragments of 56, and 31–35 kDa, which are differentially regulated by TPA, ConA, and MMP inhibitors. With the exception of the 56-kDa species, which was retained in the pellet after ultracentrifugation and thus, may be associated with membrane fragments (52), all other MT1-MMP species were soluble and thus, represent true shedding. Shedding of the 50- and 18-kDa species occurred without external stimulation, indicating that they represent a normal process of MT1-MMP turnover under basal conditions. However, exposure of cells to either TPA or ConA, two nonphysiological agents known to induce MT1-MMP expression and pro-MMP-2 activation (29, 50, 59), resulted in increased levels of all the soluble forms in the media. Based on the protease inhibitor profile, both autocatalytic and non-autocatalytic processes are involved in MT1-MMP shedding. High affinity natural (TIMP-2 and TIMP-4, but not TIMP-1) and synthetic MMP inhibitors, known to stabilize active MT1-MMP on the cell surface by inhibiting the processing of MT1-MMP to the 44-kDa form (17, 19–21), inhibited shedding of the 18-kDa species. Additionally, the E240A-MT1 catalytic mutant enzyme, which cannot be processed to the 44-kDa species (22, 60), failed to shed this fragment. Thus, shedding of the 18-kDa species is the product of the autocatalytic processing of active MT1-MMP (57 kDa) on the cell surface, which yields a major inactive membrane-tethered species of 44 kDa (17). Consistently, TPA and ConA treatments, which promote MT1-MMP expression and processing on the cell surface, stimulate shedding of the 18-kDa fragment. The ability of cells to elicit autocatalytic shedding depends on the expression level of MT1-MMP on the cell surface and the levels and availability of TIMPs. High levels of TIMP-2 and/or presence of other TIMP-2-binding MMPs will alter the autocatalytic pathway by modifying TIMP-2 availability as shown in MDA-MB-231 cells, which, as opposed to HT1080 cells, do not produce MMP-2; therefore, the autocatalytic shedding (release of the 18-kDa species) is restricted.

A battery of protease inhibitors including metalloprotease, serine, and aspartic protease inhibitors failed to reduce the levels of the 50- and 31–35-kDa species. Additionally, these species were observed in the media of cells expressing the E240A-MT1 catalytic mutant. Thus, production of these soluble MT1-MMP fragments is a non-autocatalytic event. Interestingly, TPA and ConA, which promote autocatalytic processing, enhanced the levels of these species, suggesting an additional level of regulation by these agents (50). The identity of the protease(s) responsible for the non-autocatalytic shedding of MT1-MMP remains to be determined. Our evidence and previous evidence (23, 24) suggest that, in the case of the 50-kDa species, the protease(s) must cleave within the juxtamembrane (stem) region of MT1-MMP causing the release of the entire ectodomain. Consistently, the soluble 50-kDa species was able to form a complex with TIMP-2, in agreement with early studies (24). Whether cleavage at the stem region takes place at the cell surface or intracellularly, as shown with MT5-MMP (18), remains to be determined. However, it is unlikely that a furin-like enzyme is involved in this process because, in contrast to MT5-MMP, a specific furin-recognition motif was not found in the ectodomain of MT1-MMP (18). Based on the pattern of MT1-MMP forms present on the cell surface (the 57- and 44-kDa species) and the high levels of the 18-kDa species, as determined by the immunoprecipitation experiments, the non-autocatalytic pathway is likely to comprise a minor aspect of the shedding process. However, this process may produce functional enzyme fragments, such as the 50-kDa species (24), which would promote pro-MMP-2 processing, as demonstrated here with the conditioned media of BS-C-1 cells expressing MT1-MMP, and as shown previously in gelatin zymography assays (24). Thus, soluble ectodomain fragments with catalytic activity may extend MT1-MMP-dependent proteolysis beyond the cell surface environment by promoting the hydrolysis of a variety of substrates including extracellular matrix components (4). In addition, these fragments by binding TIMP-2 may deprive the membrane-tethered enzyme of inhibitor regulation.

An interesting observation of this study was the appearance of a 31–35-kDa soluble species that was induced either by ConA treatment or high levels of TIMP-2 or marimastat. The appearance of the 31–35-kDa species in the presence of TIMP-2 or marimastat correlated with a decrease in the levels of the 18-kDa species, suggesting the possibility that the formation of these species may be related. For example, it is possible that, in addition to the autocatalytic shedding, there is a non-MMP-dependent shedding mechanism that releases the 31–35-kDa fragment, which in turn is processed to the 18-kDa species via a metalloprotease-dependent process, as suggested by the accumulation of the 31–35-kDa species in the presence of TIMP-2 or marimastat. Alternatively, TIMP-2 binding to the 31–35-kDa species may prevent a non-metalloprotease from accessing the Ala^{255}-Ile^{256} site, thus resulting in accumulation. Indeed, the 31–35-kDa species binds TIMP-2, albeit with an apparent low affinity. Another possibility is that the accumulation of the 31–35-kDa species in the presence of TIMP-2 or marimastat represents shedding of MT1-MMP catalytic domain-inhibitor complexes. Stabilization of the membrane-anchored enzyme by formation of enzyme-inhibitor complexes (17) may induce conformational changes, which may predispose the enzyme to a non-metalloprotease-mediated ectodomain shedding. However, the lack of a readily detectable counterpart to the 31–35-kDa species on the plasma membrane suggests the possibility that this fragment(s) is not a shedding product of the membrane-bound enzyme but a result of the turnover of the larger MT1-MMP soluble species, like the 50-kDa species, via a TIMP-2-insensitive process. Structural data and studies in cellular systems with defined proteolytic backgrounds will help to distinguish between these possibilities. Although the potential contribution of the 31–35-kDa species to the formation of the 18-kDa species cannot be disregarded, this is likely to be minimal when compared with the formation of the 18-kDa species generated by the autocatalytic processing of MT1-MMP on the cell surface. Taken together, these observations further underscore the complexity of the MT1-MMP shedding process and the unexpected consequences that TIMP-2 and synthetic MMP inhibitors may have on the regulation of MT1-MMP on the cell surface, as we have previously documented (20, 21).

Previous studies reported that the cytosolic domain of MT1-MMP plays a role in the stabilization of MT1-MMP on the cell surface by altering the rate of enzyme internalization (58) and is also involved in enzyme homodimerization (60), a process thought to favor autocatalytic turnover (61). Here we have shown that the pattern of MT1-MMP shedding was essentially unaltered in enzymes lacking the cytosolic domain. This finding suggests that homotypic physical interactions mediated by the cytosolic domain (60) are not essential for MT1-MMP autocatalytic and non-autocatalytic shedding.

The autocatalytic pathway of MT1-MMP shedding concludes with the release of an 18-kDa fragment that extends from Tyr^{112} to Ala^{255}, 29 amino acid residues upstream of the Gly^{285} displayed at the N terminus of the membrane-tethered 44-kDa species (17). Therefore, shedding of the 18-kDa fragment would require cleavage at both the Ala^{255}-Ile^{256} and the Gly^{284}-Gly^{285} peptide bonds. The computational model of MT1-MMP shows that the Ala^{255}-Ile^{256} peptide bond near the methionine turn is sheltered and thus is less accessible to proteolysis. This sug-

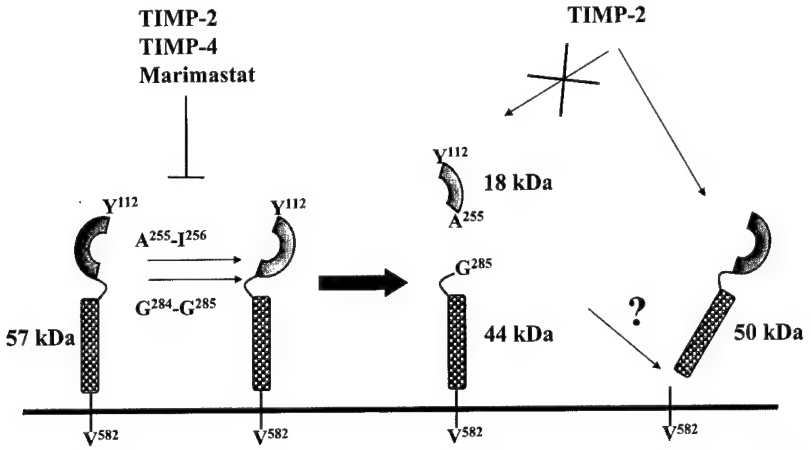

FIG. 9. **Schematic representation of the autocatalytic and non-autocatalytic shedding of MT1-MMP.** A membrane-inserted active MT1-MMP (57 kDa) undergoes intermolecular processing by a sequential cleavage at the Gly^{284}-Gly^{285} site at the beginning of the hinge region and at the Ala^{255}-Ile^{256} site within the catalytic domain. These events result in the shedding of a soluble 18-kDa species comprising Tyr^{112}–Ala^{255} and yield a residual membrane-tethered species of 44 kDa comprising Gly^{285}–Val^{582}, both of which are inactive enzyme fragments and incapable of binding TIMP-2. The autocatalytic shedding is inhibited by the natural MT1-MMP inhibitors, TIMP-2 and TIMP-4, and high affinity synthetic MMP inhibitors like marimastat. Consequently, these inhibitors stabilize active MT1-MMP on the cell surface (16, 17, 19, 21, 62). In the non-autocatalytic shedding of MT1-MMP (on *right side* of figure), a yet unknown protease releases the ectodomain (~50 kDa, no structural information yet), which possess catalytic activity (24). This species can bind TIMP-2. Not shown here is the minor ~31–35-kDa soluble fragment, which may be related to these pathways of shedding. *Single-letter* amino acid codes are used.

gests that cleavage at the Gly^{284}-Gly^{285} peptide bond, which is on the surface, is likely to precede that at the Ala^{255}-Ile^{256} site. However, at present, it is unclear whether cleavage at the Gly^{284}-Gly^{285} site predisposes for hydrolysis at the second Ala^{255}-Ile^{256} site. Data from the recombinant 21-kDa fragment, which contains the Ala^{255}-Ile^{256} site but ends at Gly^{284}, clearly indicates that disruption of the Gly^{284}-Gly^{285} site does not disturb the integrity and functionality of the Ala^{255}-Ile^{256} peptide bond. Indeed, we have shown that the 21-kDa fragment is stable and catalytically competent. Furthermore, the 21-kDa fragment was not hydrolyzed at the Ala^{255}-Ile^{256} site when was incubated alone or with HT1080 cells or their conditioned media (data not shown). We posit that the sequence of events leading to the cleavage of the Ala^{255}-Ile^{256} peptide bond after cleavage at the Gly^{284}-Gly^{285} site occur only within membrane-tethered active MT1-MMP molecules. Conceivably, cleavage at the Gly^{284}-Gly^{285} site within a membrane-anchored enzyme destabilizes the structure yielding the Ala^{255}-Ile^{256} peptide bond susceptible for subsequent hydrolysis. The inhibitor profile suggests that cleavage at the Gly^{284}-Gly^{285} site is an autocatalytic event because high affinity MT1-MMP protease inhibitors like TIMP-2 and TIMP-4 (19, 53, 62) prevented formation of the 44-kDa species starting at Gly^{285} (17). Additionally, a catalytic mutant of MT1-MMP was not processed to the 44-kDa species, as previously reported (22, 60). In regard to the Ala^{255}-Ile^{256} site, the data suggest that cleavage at that site is most likely to be also autocatalytic because TIMP-1 does not prevent shedding of the 18-kDa species. In addition, if it were mediated by another metalloprotease or a serine protease, the presence of TIMP-1, marimastat, or serine protease inhibitors should have resulted in the appearance of the 21-kDa fragment extending from Tyr^{112} to Gly^{284}, which was not detected. According to the N terminus of the membrane-bound 44-kDa species (Gly^{285}) and the C-terminal end of the soluble 18-kDa fragment (Ala^{255}), shedding of the catalytic domain should proceed via an intermediate species of ~21 kDa. However, such a species could not be detected on the cell surface or in the media of cells expressing recombinant or natural MT1-MMP. A plausible explanation for the absence of a soluble 21-kDa fragment during the shedding of the 18-kDa species is that the cleavages at the Ala^{255}-Ile^{256} and Gly^{284}-Gly^{285} sites occur rapidly and sequentially and thus would preclude accumulation of a 21-kDa intermediate form and thus ends with the 18-kDa fragment as the final product. Attempts to induce accumulation of the intermediate 21-kDa species by generating A255V or A255I substitutions at the Ala^{255}-Ile^{256} cleavage site were unsuccessful as these mutants failed to undergo activation and processing, demonstrating the importance of this site for catalytic competence.[3]

The 18-kDa fragment ends just one residue upstream of the conserved Met^{257} known to be part of the methionine turn, a structural feature characteristic of all members of the MMP family and of the super family of metzincins (55). Topologically, the methionine turn is positioned near the three histidines that coordinate with the catalytic zinc ion and is on the opposite side of these residues with respect to the active site cavity. Thus, the methionine turn is thought to be critical for catalysis, based on its close proximity to the coordination site for the catalytic zinc ion. Furthermore, the side chain of Ile^{256}, upstream of Met^{257}, forms a portion of the S1′ pocket of this enzyme. The loss of the 29-amino acid fragment during the formation of the 18-kDa MT1-MMP species would by necessity truncate the S1′ pocket; hence, it has the ability to potentially impair or alter substrate binding properties. Furthermore, the proximity of the surface loop that bears the methionine residue to the TIMP-2 binding region (43) indicates that a disorder in this location would likely impair TIMP-2 binding. Our results with the 18-kDa fragment support this notion and provide experimental documentation of the importance of the methionine turn for MMP-mediated catalysis and TIMP binding. It is worth noting that, as far as we know, the proteolytic inactivation at the methionine turn as it occurs during MT1-MMP processing has not been reported for other members of the MMP family including soluble MMPs, despite the conserved nature of this motif. This suggests the possibility that MT1-MMP specifically developed a self-controlling mechanism to allow the enzyme to function principally as a membrane-anchored protease, and any perturbation in its cellular localization would result in specific enzyme inactivation. This may explain why a transmembrane-deleted soluble MT1-MMP expressed in HT1080 cells was processed at the Ala^{255}-Ile^{256} site,

[3] P. Osenkowski and R. Fridman, manuscript in preparation.

possibly by the endogenous MT1-MMP (15). Together, these data reveal the importance of the Ala^{255}-Ile^{256} site for the maintenance of catalytic competence in MT1-MMP. Sequence alignment of the transmembrane MT-MMPs (MT1-, MT2-, MT3-, and MT5-MMP) (26) reveals a complete homology around the A-I peptide bond and the residues near the methionine turn (depicted in Fig. 5*C*). Presently, the shedding mechanisms of the MT-MMP family members have not been completely elucidated. It would be interesting to determine whether cleavage at the conserved Ala-Ile peptide bond represents a common and specific mechanism designed to terminate MT-MMP-dependent catalysis and TIMP interactions at the cell surface.

In summary, we have identified the major soluble forms of MT1-MMP and demonstrated the complexity of MT1-MMP shedding and its regulation by natural and synthetic MMP inhibitors. Fig. 9 depicts the autocatalytic processing of MT1-MMP on the cell surface leading to the release of the inactive 18-kDa species and the non-autocatalytic shedding leading to the release of the entire ectodomain by a yet unknown protease. Inhibitors of MT1-MMP block autocatalytic shedding and thus stabilize the active enzyme on the cell surface (17). The autocatalytic shedding terminates MT1-MMP-dependent pericellular proteolysis, independently of endogenous inhibitors, by specific hydrolysis at vital conserved sites (methionine turn). On the other hand, the non-autocatalytic shedding, as represented by the 50-kDa species (24) and possibly the 31–35-kDa species (not shown in Fig. 9), may still contribute to pericellular proteolysis and partly compensate for the removal of enzyme from the cell surface by shifting the proteolytic machinery to a new front, possibly with different substrates and functional consequences. Finally, the shed ectodomain of MT1-MMP may bind TIMPs (24) and hence alter the enzyme-inhibitor balance at the cell surface and/or may play new unexpected roles (3).

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Regulation of membrane type-matrix metalloproteinases

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Pericellular proteolysis is a hallmark of tumor cell metastasis. The membrane type (MT)-matrix metalloproteinases (MMPs) constitute a distinctive group of membrane-bound MMPs that are central mediators of surface proteolytic events that regulate tumor cell motility, metastasis and angiogenesis. As membrane-tethered proteases, the MT-MMPs exhibit unique regulatory mechanisms and interactions with metalloproteinase inhibitors and other relevant molecules. This review will focus on new emerging information on the mechanisms that regulate MT-MMP processing, activity and inhibition, and their significance for enzyme function in the tumor microenvironment.

Key words: matrix metalloproteinases / proteases / tumor invasion / stroma

Introduction

Tumor progression to the malignant phenotype is greatly dependent on the permissive action of the microenvironment. From angiogenesis to inflammation to metastasis, the interactions of tumor cells with their microenvironment provide many of the essential factors that will impact the fate of the tumor cells. One critical factor regulated by the tumor microenvironment is the production of specific proteolytic enzymes and protease inhibitors capable of altering the immediate pericellular milieu. The matrix metalloproteinases (MMPs) represent a large family of proteolytic enzymes regulated by tumor–stromal interactions that play key roles in cancer progression, promoting proliferation, angiogenesis and tumor metastasis.[1] The versatile and ubiquitous expression of MMPs in many tumor tissues exemplifies the evolutionary advantage that these enzymes confer on the neoplastic process. The MMPs' contribution to the malignant process derives from their ability to promote the degradation of a variety of biologically relevant molecules that are not limited to extracellular matrix (ECM) components but includes a growing family of MMP substrates, among them cytokines, growth factor receptors, and cell–cell and cell–matrix adhesion molecules.[2] In tumor tissues, MMP production is a contribution of both tumor and stromal cells.[3] However, MMP action must occur at the immediate pericellular space to effectively influence cellular activities. To achieve their full potential in pericellular proteolysis, the members of the MMP family evolved into secreted and membrane-tethered multidomain enzymes by incorporating distinct domains that facilitate binding to ECM components and surface molecules in the case of soluble MMPs and unique domains that anchor the enzyme to the cell surface, in the case of the MT-MMPs. New emerging data show that the MT-MMPs are major modifiers of the pericellular environment and key regulators of tumor cell behavior. This review will focus on the unique mechanisms that regulate MT-MMP function and inhibition and their significance for tumor proteolysis.

MT-MMPs and cancer

The MT-MMPs are a relatively new subfamily of membrane-anchored MMPs, which as of today includes six members: MT1-,[4,5] MT2-,[6] MT3-,[7] MT4-,[8] MT5-,[9,10] and MT6-MMP[11] (Table 1). These enzymes are highly expressed in almost all types of human cancers[12–20] and their expression has been associated with malignant parameters.[13,21–25] Consistent with their high expression in tumors, experimental *in vitro* and *in vivo* studies demon-

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strated the importance of MT-MMPs in promoting cell migration,[26–28] invasion,[29–32] experimental metastasis[33] and angiogenesis.[34,35] The role of MT-MMPs in invasion is tightly linked to the integrity of the membrane anchoring domains and the cytoplasmic tail. In the case of MT1-MMP, deletion of the transmembrane domain (TMD)[30,31,34,36] or deletions of and mutations at the cytoplasmic tail[31,37] impair *in vitro* cell invasion. MT1-MMP regulation of cell migration is not related exclusively to its catalytic activity against ECM components. Recent evidence indicates that MT1-MMP cleaves the hyaluronate receptor CD44 and, as a consequence, cells become migratory.[28] Furthermore, cleavage by MT1-MMP of tissue transglutaminase, an integrin-binding adhesion co-receptor, inhibits migration on fibronectin but enhances migration on collagen.[27] Together, these data suggest that the MT-MMPs by means of their specific cell surface localization and substrate profile allow cells to modify the necessary molecular machinery that controls the migratory and invasive phenotype.

An overview of the MT-MMP family

Structurally, the MT-MMPs possess the five basic characteristic domains of most MMPs, namely, (i) a signal sequence, (ii) a prodomain that maintains latency, (iii) a zinc-containing catalytic domain, (iv) a hinge region and (v) a C-terminal domain also known as the hemopexin-like domain (HLD).[38,39] However, the hallmark of the MT-MMPs is the presence of plasma membrane anchoring domains (Table 1). These domains, which extend from the HLD, consist of a sequence of ~30-amino acid stem region that is followed by a ~20-amino acid extension rich in hydrophobic residues which either (i) transverses the plasma membrane (known as the transmembrane domain (TMD)) and is followed by a cytoplasmic tail (~20 amino acids long) as in MT1-, MT2-, MT3-, and MT5-MMP[9] or (ii) forms a glycosylphosphatidylinositol (GPI) anchor as in MT4- and MT6-MMP.[40,41]

The prodomain of MT-MMPs contains a ~10-amino acid insert ending with a stretch of conserved basic residues (RXKR). This motif constitutes a cleavage site for enzymes of the pro-protein convertase family of serine proteases such as furin and is thought to play a role in pro-MT-MMP activation.[4,9,42] However, the proteases responsible for activation have not been clearly defined and both convertase[42–44] and non-convertase-dependent mechanisms[37] for activation have been described. Moreover, the need of prodomain cleavage for MT1-MMP activity has been questioned.[45] Recent functional and sequence analysis studies suggested the possibility of existence of a second set of basic motifs in pro-MT1-MMP that could serve as an alternate convertase-recognition site.[44]

Like all MMPs, the MT-MMPs are inhibited by the tissue inhibitors of metalloproteinases (TIMPs) family which includes four members (TIMP-1, TIMP-2, TIMP-3 and TIMP-4). However, unlike soluble MMPs, the MT-MMPs exhibit significant differences in affinities for the various TIMPs (Table 1). For example, MT1-,[46] MT2-,[47] MT3-[48] and MT5-MMP[10] are practically not inhibited by TIMP-1 but are efficiently inhibited by TIMP-2 and TIMP-3. MT1-MMP is also inhibited by TIMP-4.[49] Although exhibiting a higher affinity for TIMP-2, MT2-,[47] MT4-[50] and MT6-MMP[51] are inhibited by TIMP-1.

The MT-MMPs, with the exception of MT4-MMP, are classical ECM-degrading endopeptidases, and as such they exhibit a broad spectrum of substrate specificity degrading one or more ECM components (Table 1). It is noteworthy that MT1-MMP hydrolyzes native collagen I into 3/4–1/4 fragments in a typical collagenase fashion.[52,53] This activity has been suggested to be a major cause for the severe phenotype exhibited by MT1-MMP-deficient mice.[35,54] MT1-, MT4-, and MT6-MMP can also hydrolyze fibrinogen and fibrin.[34,50,51] Perhaps the most interesting aspect of the MT-MMP substrate profile is the emerging family of non-ECM proteins that is susceptible to their action. As shown in Table 1, proteins as diverse as pro-tumor necrosis factor-α,[50] CD44,[28] α1-proteinase inhibitor, α2-macroglobulin,[48] myelin inhibitory protein[55] and tissue transglutaminase[27] were all shown to be targets of MT-MMP activity. This underlies the broad functional relevance of MT-MMPs as modulators of cellular behavior.

MT-MMPs as zymogen activators

The major physiological activators of pro-MMP-2 (gelatinase A) are members of the MT-MMP family, and in the case of MT1-MMP this process involves the action of TIMP-2. The complex of TIMP-2 with active MT1-MMP evolved as a novel surface 'receptor' for pro-MMP-2[5,56,57] [shown in Figure 1(c)]. The TIMP-2/MT1-MMP complex leaves the C-terminal portion of TIMP-2 available for the binding of the HLD of

Table 1. Major properties of MT-MMPs

	Subgroup	Substrates ECM	Non-ECM	Representative tumor expression	Inhibitor	Soluble/ shed form
MT1-MMP (MMP-14)	TMD	Col I, II, III, Gel, Fb Fn, Ln-1, Ln-5, Ng Te, Vn, Agg, DS, Per	Pro-MMP-2 Pro-MMP-13 tTG, CD44, MIP α1-PI, α2M	Breast, Cervical, Gastric, Glioblastoma, Colorectal, Prostate, Hepatocellular, Esophageal Squamous Cell	T2, T3, T4	Yes
MT2-MMP (MMP-15)	TMD	Gel, Fn, Ln-1, Ng, Te Agg, Per	Pro-MMP-2 tTG	Gliomas, Renal Cell, Bladder, Larynx, Pancreas	T1, T2, T3	NI
MT3-MMP (MMP-16)	TMD	Col III, Gel, Fn, Ln-1 Vn, Agg	Pro-MMP-2 tTG, α1-PI, α2M	Breast, Renal Cell	T2, T3	Yes
MT4-MMP (MMP-17)	GPI	Gel, Fb, Fg	Pro-TNFα	Breast	T1, T2, T3	Yes
MT5-MMP (MMP-24)	TMD	Gel, Fn, CS, DS	Pro-MMP-2	Brain	T2	Yes
MT6-MMP (MMP-25)	GPI	Col IV, Gel, Fb, Fg Fn, CS, DS	Pro-MMP-2 α1-PI	Brain	T1, T2, T3	NI

Abreviations: **Agg**, Aggrecan; **CD44**, Hyaluronan Receptor; **Col**, Collagen; **CS**, Chondroitin Sulfate; **DS**, Dermatan Sulfate; **Fb**, Fibrin; **Fg**, Fibrinogen; **Fn**, Fibronectin; **Gel**, Gelatin; **GPI**, Glycosylphosphatidyl-Inositol; **Ln**, Laminin; **MIP**, Myelin-Inhibitory Protein; **NI**, No Information; **Ng**, Nidogen; **Per**, Perlecan; **T**, Tissue Inhibitor of Metalloproteinases; **Te**, Tenascin; **TMD**, Transmembrane Domain; **TNF**, Transforming Growth Factor; **tTG**, Surface Tissue Transglutaminase; **Vn**, Vitronectin; **α1-PI**, α1-Proteinase Inhibitor; **α2M**, α2 Macroglobulin.

pro-MMP-2. The MT1-MMP/TIMP-2/pro-MMP-2 complex, referred to as the 'ternary complex', facilitates the first cleavage of the pro-MMP-2 prodomain by a neighboring TIMP-2-free active MT1-MMP [Figure 1(c)]. Full activation of pro-MMP-2 is achieved by a second cleavage event in which the intermediate MMP-2 species is autocatalytically processed to the fully active enzyme.[58] This process occurs only at low TIMP-2 concentrations relative to MT1-MMP[59] to permit availability of enough inhibitor-free MT1-MMP to initiate pro-MMP-2 activation. On the other hand, high levels of TIMP-2 inhibit activation by blocking all free MT1-MMP molecules. The ternary complex model of pro-MMP-2 activation is restricted to TIMP-2 since TIMP-3 and TIMP-4, in spite of being able to bind to the HLD of pro-MMP-2,[49,60] do not support pro-MMP-2 activation.[49,60,61] In fact, TIMP-4 prevents activation by displacing TIMP-2 from the HLD of pro-MMP-2.[49] Thus, TIMP-4 can counteract the stimulatory effect of TIMP-2 in pro-MMP-2 activation[61] [Figure 1(c)]. These findings suggest a new paradigm in the regulation of proteolysis in cancer tissues in which a balance between TIMP-2 and TIMP-4 may determine the net activity of MMP-2 generated by MT1-MMP.

Regarding other MT-MMPs, which can also activate pro-MMP-2 (Table 1), it is still unknown whether these enzymes also use TIMP-2 for the activation process. However, although the ternary complex model of pro-MMP-2 activation is kinetically highly efficient, TIMP-2 independent mechanisms of pro-MMP-2 activation by MT-MMPs may exist, which may include the involvement of the $\alpha v\beta 3$ integrin[26,62] and members of the tight junction family of proteins such as claudin.[63]

MT1-MMP also promotes the activation of pro-collagenase-3 (MMP-13),[64] a collagenolytic MMP that was identified in the stroma of breast carcinomas.[65] The activation of pro-collagenase-3 can be directly mediated by MT1-MMP or indirectly via MMP-2.[64] The linkage of the collagenolytic activity of MT1-MMP and collagenase-3 with the gelatinolytic activity of MMP-2 illustrates an evolutionary triumph of a proteolytic system designed to promote coordinated collagen degradation in the pericellular space. These three enzymes are expressed in tumor stroma and therefore may act in concert to facilitate tumor cell invasion.

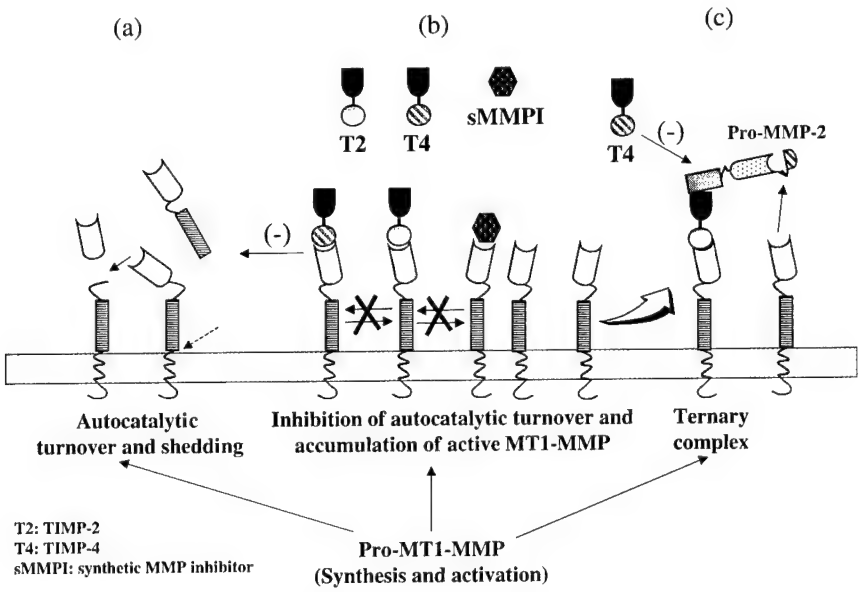

Figure 1. Role of TIMPs and synthetic MMPIs in MT1-MMP processing and function. (a) In the absence of inhibitors, autocatalytic processing of active MT1-MMP results in shedding of the ectodomain and generation of an inactive membrane-tethered form. (b) TIMP-2, TIMP-4, and synthetic MMPIs bind active MT1-MMP inhibiting autocatalytic turnover. As a consequence, active MT1-MMP accumulates on the cell surface as cells continue to synthesize and activate pro-MT1-MMP. (c) The accumulation of active MT1-MMP contributes to ternary complex formation enhancing pro-MMP-2 activation by a TIMP-2-free MT1-MMP. TIMP-4 cannot form a ternary complex and inhibits the effect of TIMP-2 on pro-MMP-2 activation.

Processing of active MT-MMPs. A new paradigm in protease regulation

The MT-MMPs developed a unique mechanism of regulation in which the active enzyme undergoes a series of processing events, either autocatalytic[66–68] or mediated by other proteases[9,69,70] that control the activity and nature of enzyme species at the cell surface and at the pericellular space. Most of the information on enzyme processing has been gathered with MT1-MMP, and thus the forthcoming schemes relate mostly to this enzyme. The processing of active MT1-MMP (57 kDa) is mostly an autocatalytic intermolecular event that results in the generation of an inactive membrane-tethered form of 44 kDa and in the shedding of the catalytic domain[31,67,68] [Figure 1(a)]. The shedding may also include release of the entire extracellular extension of MT1-MMP comprising both the catalytic domain and the HLD.[70] While this process would terminate activity on the plasma membrane independently of exogenous inhibitors, the shed catalytic domain may contribute to pericellular proteolysis. This process may compensate for the removal of enzyme from the cell surface by shifting the proteolytic machinery to a new front, possibly with different substrates and functional consequences. The shed ectodomains of MT-MMPs may also bind exogenous TIMPs and hence alter the enzyme–inhibitor balance at the cell surface.

In the case of MT1-MMP, the interaction of the active enzyme with either TIMP-2, TIMP-4 or synthetic MMP inhibitors (MMPIs) inhibits processing. As a result of this inhibition and continuous synthesis and activation of pro-MT1-MMP, the cells accumulate the 57 kDa active MT1-MMP species on the cell surface[67,68] [Figure 1(b)], which can generate additional pro-MMP-2 'receptors' by binding TIMP-2, thereby facilitating activation [Figure 1(c), right open arrow]. Thus, the inhibition of MT1-MMP processing generates a pool of active enzyme on the cell surface that supports ternary complex formation. Indeed, synthetic MMPIs, which inhibit MT1-MMP processing but cannot form a pro-MMP-2 'receptor', can further enhance the activation of pro-MMP-2 by MT1-MMP in the presence of TIMP-2 [Figure 1(b) and 1(c)].[71] Interestingly, TIMP-4, as opposed to the synthetic MMPIs, is unable to work synergistically with TIMP-2 in pro-MMP-2 activation in spite of its ability to inhibit MT1-MMP processing [Figure 1(b)],[71] possibly due to the high-affinity binding of the MT1/TIMP-4 complex,[49] which may not be displaced by TIMP-2.

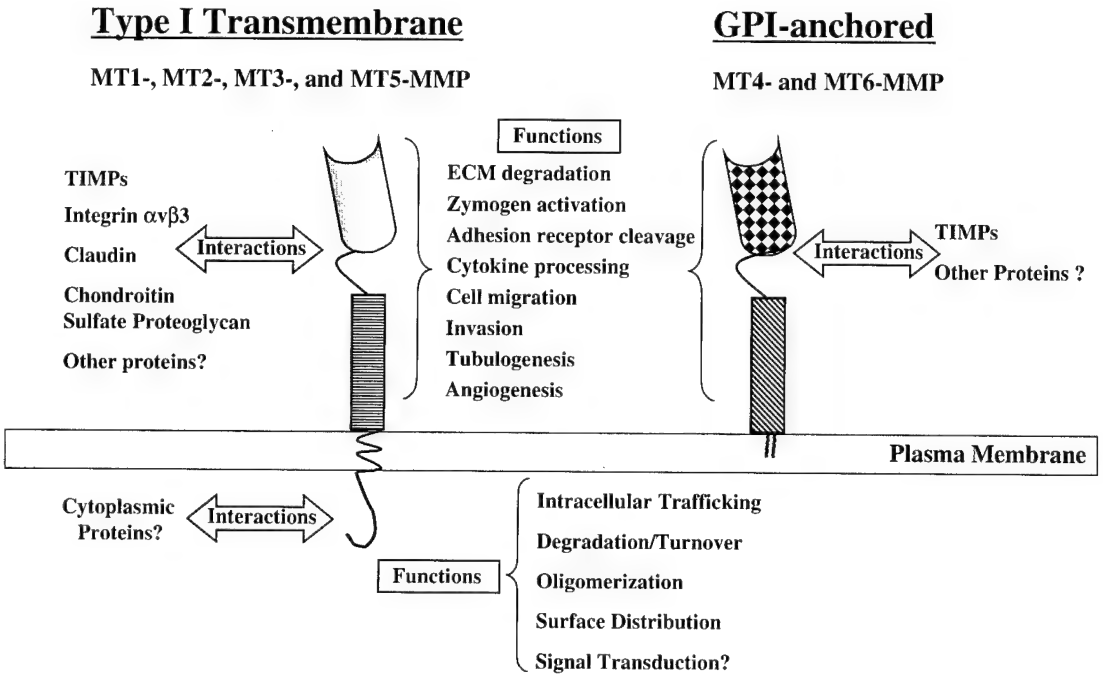

Figure 2. Multiple functions and interactions of MT-MMPs. The MT-MMPs, sub-classified as Type I transmembrane- and GPI-anchored MMPs, interact with a variety of molecules through their ectodomains. Several functions for the ectodomains were reported, as indicated. The cytoplasmic tails of the MT-MMPs with a TMD play roles in several processes and possess the potential to interact with cytoplasmic proteins.

The inhibition of autocatalytic processing may also positively influence the overall activity of MT1-MMP against a variety of pericellular substrates by stabilizing the enzyme on the cell surface. In tumor tissues, the effects of MT1-MMP stabilization in pro-MMP-2 activation and possibly in ECM degradation may contribute to the overall process of tumor cell invasion. Thus, under certain conditions, the inhibition of processing of MT1-MMP and possibly of other MT-MMPs as well, by natural or synthetic MMPIs, may produce undesirable consequences. In this regard, it is noteworthy that certain invasive tumors express high levels of stromal TIMP-2, which were found to be associated with malignant parameters.[72–76] The stromal production of TIMP-2 may represent a mechanism to counteract MMP-mediated proteolytic activity. However, given the role of TIMP-2 in MT1-MMP function, the production of TIMP-2 under certain conditions may stimulate MT1-MMP activity. Likewise, the inadequate inhibition of MT1-MMP by synthetic MMPIs may produce undesirable side effects due to the enhancement of cell surface activity as a consequence of enzyme stabilization. This complex regulation of MT1-MMP activity emphasizes the importance of understanding the molecular mechanisms and consequences of enzyme inhibition, which clinically pose new challenges for the design of novel MMP inhibitors.

Multiple functions and interactions of MT-MMPs

The MT-MMPs are emerging as major regulators of cell surface proteolysis. New interactions of MT-MMP domains with a distinctive array of cellular proteins that influence cellular behavior are being described. These interactions include associations with TIMPs, cytoplasmic and cytoskeletal proteins and cell–cell and cell–matrix receptors. Functionally, the MT-MMPs mediate pericellular proteolysis through direct ECM modification and indirectly through initiation of zymogen activation cascades and specific hydrolysis of key surface molecules (Figure 2). We can predict that the versatility of the MT-MMP molecules, some spanning from the intracellular to the extracellular milieu, will continue to supply an array of unexpected and exciting discoveries, perhaps equivalent only to that encountered with surface signaling molecules. Ultimately, the

complexity of the molecular interactions and the processes regulating MT-MMP action will have to be considered for the design of new anti-MMP therapies to be effective. Hopefully, the discovery of new MT-MMP functions and interacting molecules will also bring novel targets for intervention, which may have an impact in cancer therapy.

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TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type 1)-MMP

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The matrix metalloproteinase (MMP)-2 has a crucial role in extracellular matrix degradation associated with cancer metastasis and angiogenesis. The latent form, pro-MMP-2, is activated on the cell surface by the membrane-tethered membrane type 1 (MT1)-MMP, in a process regulated by the tissue inhibitor of metalloproteinase (TIMP)-2. A complex of active MT1-MMP and TIMP-2 binds pro-MMP-2 forming a ternary complex, which permits pro-MMP-2 activation by a TIMP-2-free neighbouring MT1-MMP. It remains unclear how MMP-2 activity in the pericellular space is regulated in the presence of TIMP-2. To address this question, the effect of TIMP-2 on MMP-2 activity in the extracellular space was investigated in live cells, and their isolated plasma membrane fractions, engineered to control the relative levels of MT1-MMP and TIMP-2 expression. We show that both free and inhibited MMP-2 is detected in the medium, and that the net MMP-2 activity correlates with the level of TIMP-2 expression. Studies to displace MT1-MMP-bound TIMP-2 in a purified system with active MMP-2 show minimal displacement of inhibitor, under the experimental conditions, due to the high affinity interaction between TIMP-2 and MT1-MMP. Thus inhibition of MMP-2 activity in the extracellular space is unlikely to result solely as a result of TIMP-2 dissociation from its complex with MT1-MMP. Consistently, immunoblot analyses of plasma membranes, and surface biotinylation experiments show that the level of surface association of TIMP-2 is independent of MT1-MMP expression. Thus low-affinity binding of TIMP-2 to sites distinct to MT1-MMP may have a role in regulating MMP-2 activity in the extracellular space generated by the ternary complex.

Key words: gelatinase, matrix metalloproteinase, protease, protease inhibitor, zymogen.

INTRODUCTION

Matrix metalloproteinase (MMP)-2 (also called gelatinase A) is a key member of the MMP family of zinc-dependent endopeptidases which has been associated with many pathological conditions, particularly cancer metastasis and angiogenesis [1–5]. The activation of the zymogen form of MMP-2 (pro-MMP-2) is a cell-surface event that is mediated by members of the membrane-type (MT) subfamily of MMPs [6], and MT1-MMP (MMP-14) was the first MT-MMP identified as a major physiological activator of pro-MMP-2 [7]. The activation of pro-MMP-2 by MT1-MMP evolved to incorporate in its process the tissue inhibitor of metalloproteinase (TIMP)-2, a member of the TIMP family of MMP inhibitors. While TIMP-2 inhibits active MT1-MMP and MMP-2 by binding to the active site of the enzymes via its N-terminal inhibitory region, it can also form a non-covalent complex with pro-MMP-2 by binding to the haemopexin-like domain of the zymogen via its C-terminal domain [8–14]. It has been shown that the simultaneous binding of TIMP-2 to active MT1-MMP via its inhibitory N-terminal domain, and to pro-MMP-2 via its C-terminal region, yields ternary complexes on the cell surface that permits pro-MMP-2 activation by a TIMP-2-free neighbouring MT1-MMP molecule, which hydrolyses the Asn^{37}–Leu^{38} peptide bond of pro-MMP-2 [12,15,16]. This process generates an inactive intermediate MMP-2 form that is subsequently cleaved at the Asn^{80}–Tyr^{81} peptide bond by a fully active MMP-2, in an intermolecular autocatalytic event, which leads to full activation [17]. In cultured cells, active MMP-2 (62 kDa) is detected in the supernatant and in the cell layer, indicating that the 62 kDa species dissociates from the ternary complex upon activation. However, it is unclear whether the 62-kDa species of MMP-2 detected in the medium is free or inhibited (in complex) by TIMP-2. A previous study, using isolated plasma membranes (PMs) of concanavalin A (ConA)-treated human uterine cervical fibroblasts expressing natural MT1-MMP, showed that the active MMP-2 in the medium was in a complex with TIMP-2, and was thus devoid of catalytic activity [18]. In another study, conducted on breast carcinoma cells stably transfected to express human MT1-MMP, MMP-2 was shown to dissociate from the cell surface free of TIMP-2 [19]. Thus the effects of TIMP-2 on the MMP-2 activity generated by the ternary complex system of pro-MMP-2 activation remain unclear. To investigate the interaction of MMP-2 with TIMP-2 after activation by MT1-MMP, we determined the net proteolytic activity of MMP-2 in a cellular system designed to control the relative levels of TIMP-2 and MT1-MMP expression.

MATERIALS AND METHODS

Cells and recombinant viruses

Non-malignant monkey kidney epithelial BS-C-1 (CCL-26) cells were obtained from the American Type Culture Collection

Abbreviations used: APMA, *p*-aminophenylmercuric acetate; ConA, concanavalin A; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; HRP, horseradish peroxidase; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$, (7-methoxycoumarin-4-yl)acetyl-L-prolyl-L-glycyl-L-leucyl-[N$_3$-(2,4-dinitrophenol)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide; MT1-MMP, membrane type 1-MMP; MT1-MMP$_{cat}$, catalytic domain of MT1-MMP; NP40, Nonidet P40; pAb, polyclonal antibody; pfu, plaque-forming unit(s); PM, plasma membrane; TIMP, tissue inhibitor of metalloproteinase.

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(A.T.C.C., Manassas, VA, U.S.A.), and were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % foetal bovine serum (FBS) and antibiotics. HeLa S3 cells were obtained from the ATCC (CCL-2.2), and were grown in suspension in minimal essential medium (Spinner modification; Quality Biologicals, Gaithersburg, MD, U.S.A.), supplemented with 5 % (v/v) horse serum. All other tissue-culture reagents were purchased from Invitrogen (Carlsbad, CA, U.S.A.). The production of the recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase has been described by Fuerst et al. [20]. Recombinant vaccinia viruses expressing either pro-MT1-MMP (vT7-MT1) or TIMP-2 (vSC59-T2) were obtained by homologous recombination, as previously described [10,21].

Recombinant proteins and antibodies

Human recombinant pro-MMP-2 and TIMP-2 were expressed in HeLa S3 cells infected with the appropriate recombinant vaccinia viruses, and were purified to homogeneity, as described in [22]. Pro-MMP-2 was activated by incubation with *p*-aminophenylmercuric acetate (APMA), as described in [23], and the MMP-2 concentration was determined by titration against a TIMP-2 solution of known concentration. The catalytic domain of MT1-MMP (MT1-MMP$_{cat}$), expressed in bacteria, was obtained from Calbiochem (La Jolla, CA, U.S.A.). The anti-MMP-2 mouse monoclonal antibody (mAb) CA-801, the anti-TIMP-2 mAb CA-101 and the rabbit polyclonal antibodies (pAb) 437 and 160, against the haemopexin-like and catalytic domains of MT1-MMP respectively, have been described previously [21,24,25].

Cell infection and PM isolation

BS-C-1 cells in six-well plates were co-infected with vTF7-3 vaccinia virus [5 plaque-forming units (pfu)/cell], vT7-MT1 (5 pfu/cell), and increasing pfu/cell (0–5) of vSC59-T2 virus, in 0.5 ml/well of infection medium (DMEM supplemented with 2.5 % FBS and antibiotics), for 45 min at 37 °C. The virus-containing medium was aspirated and replaced with fresh infection medium for an additional incubation of 16 h, at 37 °C. To isolate PM fractions, BS-C-1 cells in 150 mm diameter tissue-culture dishes were co-infected with vTF7-3 vaccinia virus (5 pfu/cell) and either constant vT7-MT1 vaccinia virus (5 pfu/cell) and increasing vSC59-T2 vaccinia virus (0–5 pfu/cell) or increasing vT7-MT1 virus (0–5 pfu/cell) and constant vSC59-T2 virus (0.05 pfu/cell), in 25 ml/dish of infection medium, for 45 min at 37 °C. After 16 h incubation in fresh infection medium, the cells were subjected to subcellular fractionation to isolate the PM fraction, as described in [21].

Pro-MMP-2 activation by cells and PM fractions

BS-C-1 cells in six-well plates were infected to co-express MT1-MMP and TIMP-2, as described above. At 16 h after infection, the medium was aspirated and replaced with serum-free Opti-MEM (modified Eagle's medium), without Phenol Red, supplemented with 50 nM of purified human recombinant pro-MMP-2. At various times ($\leqslant$ 6.5 h), medium was removed for analysis of MMP-2 activation by gelatin zymography, immunoblotting, and by a proteolytic activity assay, using the fluorescence-quenched substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$ {(7-methoxycoumarin-4-yl)acetyl-L-prolyl-L-glycyl-L-leucyl-[N$_3$-(2,4-dinitrophenol)-L-2,3-diaminopropionyl]-L-alanyl-L-arginine amide; Peptides International, Louisville, KY, U.S.A.} [26], as described in [27]. Briefly, 20 μl medium aliquots were added to 2 ml of a 7 μM solution of the fluorogenic substrate in a buffer consisting of 50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM CaCl$_2$, 0.01 % (v/v) Brij-35 and 1 % (v/v) DMSO (buffer R). Substrate hydrolysis was monitored at 25 °C, using a Photon Technology International (PTI) spectrofluorometer, at excitation and emission wavelengths of 328 and 393 nm respectively. After approx. 6.5 h, the remaining medium was collected, the cells were rinsed with cold PBS, and were lysed with cold lysis buffer [25 mM Tris/HCl (pH 7.5), 1 % Igepal CA-630, a non-ionic detergent from Sigma, and 100 mM NaCl] containing protease inhibitors (one pellet of Complete Mini, EDTA-free protease inhibitor cocktail from Roche Diagnostics, Mannheim, Germany, in 10 ml of buffer). The medium samples were centrifuged at 500 ***g***, for 5 min at 4 °C, to remove cell debris. The lysates were centrifuged at 9500 ***g*** for 15 min at 4 °C. To ascertain the extent of TIMP-2–MMP-2 complex formation, the medium was incubated overnight at 4 °C with gelatin immobilized on crossed-linked 4 % beaded agarose [50 μl of a 50 % slurry in collagenase buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM CaCl$_2$, 0.02 % (v/v) Brij-35]. After centrifugation at 9500 ***g*** for 5 min, the supernatants were collected, and the beads (pellet) were subjected to three washing/centrifugation steps with collagenase buffer (200 μl). The beads were mixed with reducing Laemmli sample buffer and were boiled for 5 min. The supernatants were subjected to immunoblot analysis for TIMP-2 and pro-MMP-2/MMP-2. Lysate fractions were analysed for pro-MMP-2 activation by gelatin zymography, and for MT1-MMP and TIMP-2 expression by immunoblot analysis. Pro-MMP-2 activation by PM fractions was monitored in reaction mixtures containing pro-MMP-2 (50 nM) and PM (0.150 μg/ml) in collagenase buffer. At various times ($\leqslant$ 7 h), aliquots of the reaction mixtures were subjected to gelatin zymography and immunoblot analysis, and MMP-2 proteolytic activity was assayed with the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$, as described above.

Displacement of MT1-MMP-bound TIMP-2

To examine the displacement of membrane-bound TIMP-2 by MMP-2, increasing concentrations of PM fractions, derived from BS-C-1 cells infected to co-express varying MT1-MMP/TIMP-2 ratios, were incubated (1 h, 37 °C) with 5 nM MMP-2 in collagenase buffer. The remaining MMP-2 activity was measured with the synthetic peptide substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$, as described above. To follow the ability of MMP-2 to displace TIMP-2 bound to MT1-MMP in a purified system, 130 nM MT1-MMP$_{cat}$ was incubated (1 h, 37 °C) with 100 nM TIMP-2, to ensure that all the TIMP-2 was complexed with MT1-MMP. Complete MT1-MMP$_{cat}$ inhibition by TIMP-2 was confirmed with the fluorogenic substrate MOCAcPLGLA$_2$pr-(Dnp)AR-NH$_2$. Displacement experiments were carried out either by adding MMP-2 (0.8 nM) to 2 ml of buffer R, containing the pre-formed MT1-MMP$_{cat}$–TIMP-2 complex (0.6 nM) and the fluorogenic substrate (7 μM), and monitoring substrate hydrolysis over 20 min, or by pre-incubating MMP-2 (5 nM) with increasing MT1-MMP$_{cat}$–TIMP-2 complex (0–20 nM) for 1 h, at 37 °C, in a total volume of 100 μl in buffer R, and measuring the remaining MMP-2 activity with the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$, as described above. The activity due to MT1-MMP$_{cat}$ added in excess of TIMP-2 was measured in a reaction mixture, run in parallel, containing the same complex concentration but no MMP-2, and subtracted from that obtained in the presence of the enzyme.

Gelatin zymography and immunoblot analysis

Gelatin zymography was performed as previously described in [28]. The samples for immunoblot analysis were subjected to reducing SDS/PAGE, followed by transfer to nitrocellulose membranes. The transferred proteins were developed with pAb 437 and pAb 160 against MT1-MMP, mAb CA-101 against TIMP-2, or mAb CA-801 against pro-MMP-2/MMP-2. Horseradish peroxidase (HRP)-labelled anti-(rabbit MT1-MMP), and anti-(mouse pro-MMP-2/MMP-2 and TIMP-2) IgG (ImmunoPure® from Pierce, Rockford, IL, U.S.A.) were the secondary antibodies used. Detection was performed using SuperSignal®, West Pico or Femto sensitivity, enhanced chemiluminescent substrate for HRP according to the manufacturer's (Pierce) instructions.

Surface biotinylation

BS-C-1 cells, in 150 mm diameter dishes, were co-infected with vTF7-3 (5 pfu/cell), vSC59-T2 (0.05 pfu/cell) and increasing vT7-MT1 (0–5 pfu/cell) viruses, as described above. After 18 h of incubation, the cells were washed extensively with cold PBS-CM (PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$). To each dish, 10 ml of freshly prepared 0.5 mg/ml sulpho-*N*-hydroxysuccinimide-biotin (Pierce), in PBS-CM were added. After incubation for 30 min at 4 °C, the cells were rinsed with cold PBS-CM and were incubated for 10 min at 4 °C with 50 mM NH_4Cl in PBS-CM. The cells were rinsed with PBS-CM and lysed with NP40 (Nonidet P40) buffer [25 mM Tris (pH 7.5), 100 mM NaCl and 1 % (v/v) NP40] containing protease inhibitors, as described above. The lysates were centrifuged at 9500 ***g*** at 4 °C for 13 min. The supernatants were incubated with 120 μl of a 1:1 slurry of ImmunoPure® immobilized streptavidin on agarose beads (Pierce) for 10 min at 4 °C, followed by centrifugation for 10 min at 9500 ***g***. The beads were extensively washed with NP40 buffer, treated with 4× reducing Laemmli sample buffer, and boiled. After centrifugation, the samples were subjected to immunoblot analysis.

RESULTS

TIMP-2 modulates MMP-2 activity in cells

We followed the activity of MMP-2 generated after pro-MMP-2 activation, in cells infected to express constant amounts of MT1-MMP, and increasing amounts of TIMP-2, by using a mammalian cell expression system and recombinant vaccinia viruses. Generation of active MMP-2 was monitored in the medium by zymography, immunoblot analysis, and by a proteolytic activity assay, as a function of time, after addition of exogenous pro-MMP-2 to the MT1-MMP–TIMP-2-expressing cells. As shown in Figure 1(A), the peptide-substrate assay demonstrated that the net activity of MMP-2 increased with time, and with the level (pfu/cell) of TIMP-2 virus > 0.1 pfu/cell. Higher levels of TIMP-2 virus (0.5 and 1 pfu/cell), and consequently TIMP-2 protein (Figure 1D), caused a significant inhibition of MMP-2 activity in the supernatant (Figure 1A). This inhibition of activity did not correlate with the zymographic and immunoblot analyses of MMP-2 (Figures 1B and 1C), which revealed the presence of active MMP-2 species, even at the highest levels of TIMP-2, suggesting that the decrease in activity was likely to be due to inhibition by TIMP-2. In fact, increasing concentrations of TIMP-2 were detected in the medium by immunoblot analysis (Figure 1D), and no significant amount of free TIMP-2 was measured by titration with exogenous MMP-2, at all levels of TIMP-2 virus infection (results not shown).

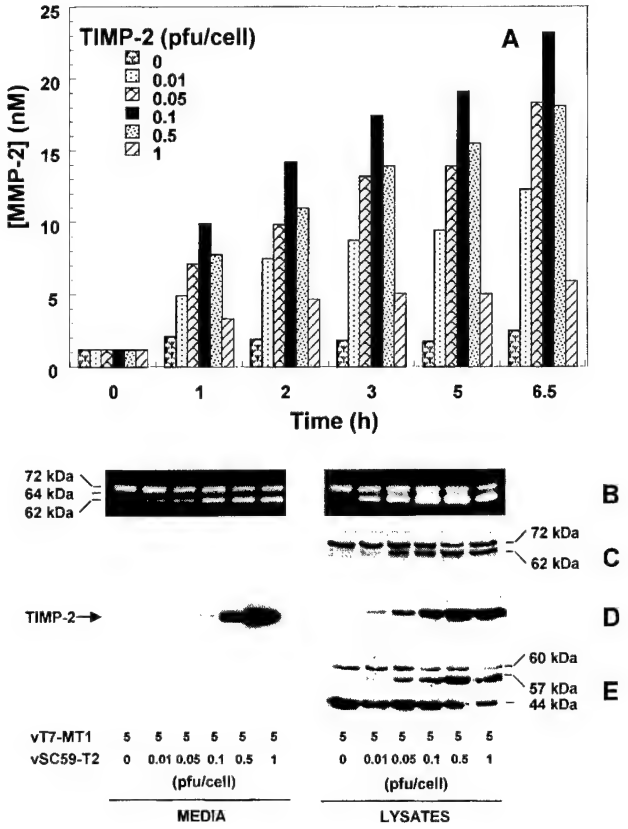

Figure 1 Effect of TIMP-2 levels on MMP-2 activity in cells

(**A**) BS-C-1 cells were co-infected to express MT1-MMP (5 pfu/cell), and increasing amounts of TIMP-2 (0–1 pfu/cell), as described in the Materials and methods section. At 16 h post-infection, the medium was aspirated and was replaced with Opti-MEM (modified Eagle's medium) containing 50 nM of purified human recombinant pro-MMP-2. At various times ($\leq$ 6.5 h), aliquots of the medium were assayed for MMP-2 proteolytic activity, using the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH_2, as described in the Materials and methods section. (**B**) After 6.5 h of incubation, medium and lysates were subjected to gelatin zymography, as described in the Materials and methods section. (**C**) Immunoblot analysis of the lysates using the mAb CA-801 against pro-MMP-2/MMP-2. (**D**) Immunoblot analysis of the medium and lysates, after 6.5 h incubation, using the mAb Ca-101 against TIMP-2. (**E**) Immunoblot of the lysates using pAb 437 against MT1-MMP.

The expression of TIMP-2 also modulated the profile of MT1-MMP forms in the cells. As shown in Figure 1(E), in the absence of TIMP-2, MT1-MMP exhibited mostly 60 and 44 kDa forms, representing the zymogen and a membrane-tethered form lacking the catalytic domain respectively [21]. Expression of TIMP-2 correlated with the presence of the 57 kDa active species of MT1-MMP, and the concomitant reduction in the 44 kDa form, consistent with the ability of TIMP-2 to prevent the autocatalytic turnover of MT1-MMP, as previously described in [21]. Thus the sustained level of pro-MMP-2 activation detected by zymography (Figures 1B and 1C) correlated with a reduced autocatalytic processing of MT1-MMP.

To ascertain whether the TIMP-2 found in the medium was free or bound to MMP-2, the medium was incubated with gelatin–agarose beads. Gelatin beads are known to bind both latent and active gelatinases, free or complexed with TIMPs. The bound fraction, comprising all free and complexed MMP-2 forms, was subjected to immunoblot analysis, whereas the unbound fraction, comprising free TIMP-2, was analysed for its ability to inhibit MMP-2 activity. As shown in Figure 2(B), increasing amounts of TIMP-2 were detected in the bound fraction, which

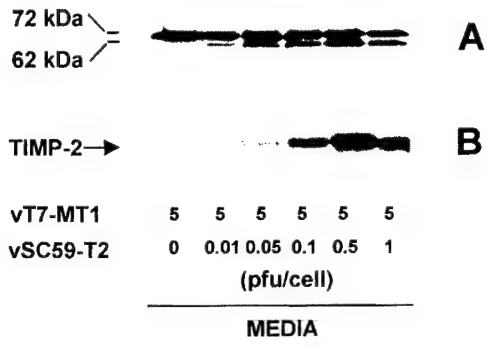

Figure 2 Effect of TIMP-2 levels in MMP-2–TIMP-2 complex formation

(**A**) After 6.5 h of incubation, the MMP-2-containing medium of BS-C-1 cells co-infected to express constant MT1-MMP and increasing TIMP-2, as described in Figure 1, was treated with gelatin–agarose beads, as described in the Materials and methods section, and the bound fraction was subjected to immunoblot analysis using mAb CA-801 against pro-MMP-2–MMP-2. (**B**) The blot was reprobed using mAb CA-101 against TIMP-2.

correlated with the extent of pro-MMP-2 activation, as revealed by the increased intensity of the 62 kDa MMP-2 form (Figure 2A). In contrast, the unbound fraction revealed no inhibitory activity after titration against active MMP-2, suggesting that the TIMP-2 released into the medium is bound to the enzyme (results not shown). Taken together, these results indicate that, although the highly regulated mechanism of pro-MMP-2 activation by MT1-MMP seems to confine proteolytic activity to the cell surface, both active and inhibited MMP-2 are found in the medium, and the level of proteolytic activity is a function of the MT1-MMP/TIMP-2 ratio expressed by the cells.

Activation of pro-MMP-2 by PM fractions

Since the infected cells secrete TIMP-2 constitutively, it was difficult to establish whether the inhibition of MMP-2 was due to TIMP-2 secreted into the medium over the time period of the experiment, or due to the dissociation of TIMP-2 originally bound to the cell surface. Therefore, to measure MMP-2 activity in the absence of secreted TIMP-2, we used, instead of live cells, the PM fraction derived from cells infected to express MT1-MMP and increasing amounts of TIMP-2. The isolated PM fractions were washed thoroughly to remove all unbound TIMP-2 and thus all the inhibitor present was expected to be bound to the PM. Immunoblot analyses of the PM fractions demonstrated the presence of MT1-MMP (Figure 3C) and TIMP-2 (Figure 3D), as expected. The PM fractions were incubated for various times with exogenous pro-MMP-2, and activation was monitored as described above. As shown in Figure 3(A), the MMP-2 activity generated by the PM fractions correlated with the amount of TIMP-2 virus used to infect the cells > 0.1 pfu/cell. However, higher levels of TIMP-2 virus caused a sharp decline in activity. In terms of MT1-MMP, pro-MMP-2 activation correlated with accumulation of active MT1-MMP (57 kDa), and with the concomitant decrease of the 44 kDa species with increasing TIMP-2 expression (Figure 3C). These results did not differ significantly from the results obtained with the live cells, suggesting that MMP-2 inhibition is likely to be mediated by TIMP-2 associated with the PM.

MMP-2 displaces surface-bound TIMP-2

The experiments described above suggested that, once activated, MMP-2 could displace cell associated TIMP-2 resulting in MMP-

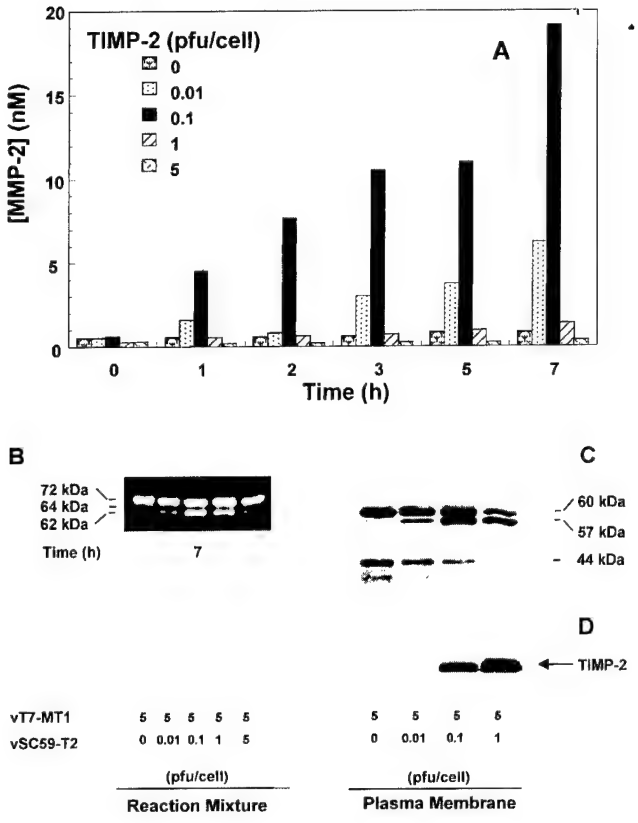

Figure 3 Effect of TIMP-2 levels in MMP-2 activity in PM fraction

(**A**) PM (0.15 μg/μl) isolated from BS-C-1 cells co-infected to express constant levels of MT1-MMP (5 pfu/cell) and increasing levels of TIMP-2, indicated by the pfu/cell of the TIMP-2-expressing virus (vSC59-T2) (0–5 pfu/cell), were incubated at 37 °C with 50 nM of pro-MMP-2, in a total volume of 200 μl of collagenase buffer. At various times ($\leqslant 7$ h), MMP-2 activity was assayed with the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$ in buffer R. (**B**) Zymographic analysis of the reaction mixtures after 7 h of incubation. (**C**) Immunoblot analysis of MT1-MMP in PM fractions with pAb 437. (**D**) Immunoblot analysis of TIMP-2 in PM fractions with mAb CA-101.

2 inhibition. To test this hypothesis, APMA-activated MMP-2 was incubated for 1 h at 37 °C with various amounts of PM fractions isolated from cells infected to express either constant levels of MT1-MMP and increasing TIMP-2 amounts, or increasing levels of MT1-MMP and constant amounts of TIMP-2. Then the remaining MMP-2 activity was measured with the fluorogenic peptide substrate. As shown in Figure 4(A), the inhibition of MMP-2 activity correlated with the level of TIMP-2 detected in the PM fractions isolated from cells expressing increasing levels of TIMP-2 (shown in the immunoblot of Figure 3D). Since MT1-MMP is known to function as a high-affinity receptor for TIMP-2 [15,29], this result suggests that MMP-2 may be displacing MT1-MMP-bound TIMP-2. To test this hypothesis, MMP-2 was added to a solution of a pre-formed MT1-MMP$_{cat}$–TIMP-2 complex in low equimolar concentrations (approx. 0.8 nM). Although both MT1-MMP$_{cat}$ and MMP-2 react with the fluorogenic substrate, MMP-2 reacts with higher catalytic efficiency, as revealed by the respective k_{cat}/K_m values (1×10^5 and 10×10^5 $M^{-1} \cdot s^{-1}$ respectively [22,25]). Thus TIMP-2 displacement from the MT1-MMP$_{cat}$–TIMP-2 complex by MMP-2 should result in a net decrease of proteolytic activity. However, no decay of activity was detected over a 20 min period, suggesting that the MT1-MMP–TIMP-2 complex cannot easily be dissociated by MMP-2 at low equimolar enzyme concentrations

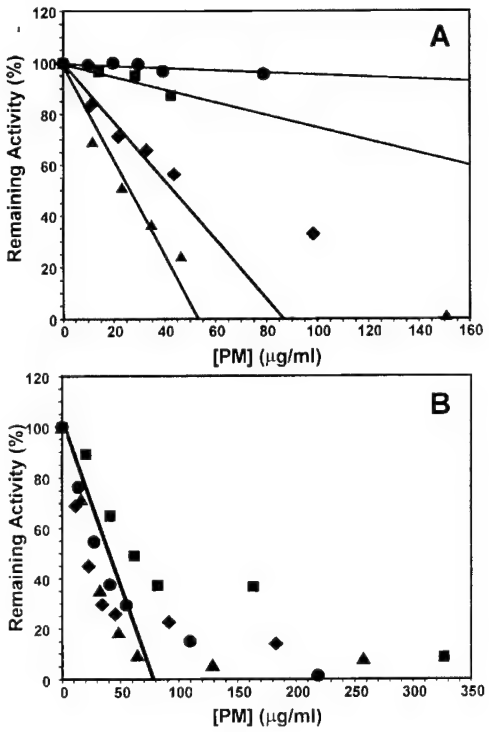

Figure 4 MMP-2 displaces TIMP-2 from PM

Recombinant active MMP-2 (5 nM) was incubated with increasing concentrations of PM fractions isolated from BS-C-1 cells co-infected with vTF7-3 (5 pfu/cell) and (**A**) vT7-MT1 (5 pfu/cell) viruses and various vSC59-T2 virus [0 (●), 0.01 (■), 0.1 (◆) and 1 (▲) pfu/cell] or (**B**) increasing vT7-MT1 [0 (●), 0.01 (■), 0.1 (◆) and 5 (▲) pfu/cell] and constant vSC59-T2 (0.05 pfu/cell) in a total volume of 50 μl, in collagenase buffer, for 1 h, at 37 °C. The remaining MMP-2 proteolytic activity after incubation was measured using the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$, as described in the Materials and methods section.

(results not shown), indicating that MMP-2 displacement of MT1-MMP-bound TIMP-2 would require high concentrations of MMP-2 and/or long incubation periods. In fact, at higher MMP-2 concentrations (5 nM) and longer incubation periods (1 h at 37 °C), a 40 % decay in MMP-2 activity with increasing MT1-MMP$_{cat}$–TIMP-2 concentrations (0–20 nM) was observed (results not shown). Of note, the decrease in proteolytic activity is likely to be larger than 40 %, since it includes the contribution of the dissociated MT1-MMP$_{cat}$. To confirm that TIMP-2 was displaced, the reaction mixtures were incubated with gelatin–agarose beads, and the free and bound fractions were subjected to immunoblot analysis. MMP-2, but not MT1-MMP$_{cat}$, was detected in the bound fraction, as expected, given the low gelatinolytic activity of MT1-MMP$_{cat}$. Increasing TIMP-2 amounts were also found in the bound fraction, indicating that MMP-2 can displace MT1-MMP-bound TIMP-2. Thus the ability of the PM fractions with increasing TIMP-2 levels to inhibit MMP-2 may not be caused solely by displacement of MT1-MMP-bound TIMP-2, but also by TIMP-2 associated with sites that are distinct from MT1-MMP. Consistently, titration of MMP-2 against increasing amounts of PM fractions derived from cells infected to express constant levels of TIMP-2 and increasing levels of MT1-MMP revealed that MMP-2 inhibition correlated with the level of TIMP-2, rather than that of MT1-MMP (Figure 4B). As shown in Figure 5(A), immunoblot analysis of the PM fractions showed that the relative amount of TIMP-2 present was independent of the level of MT1-MMP expression. Furthermore, cell-surface biotinylation of BS-C-1 cells infected to express the same levels of MT1-MMP and TIMP-2 showed similar amounts of surface-associated TIMP-2, regardless of the level of MT1-MMP expression, as shown in Figure 5(B), suggesting that TIMP-2 binds to sites other than MT1-MMP on the cell surface. Taken together, these results indicate that, although MT1-MMP has been identified as a major high-affinity TIMP-2 receptor, presumably weaker specific or non-specific TIMP-2 binding to the cell surface may modulate MMP-2 proteolytic activity in the pericellular environment.

DISCUSSION

MT1-MMP activation of pro-MMP-2 requires the participation of TIMP-2, which acts as a linker between MT1-MMP and pro-MMP-2, to generate a ternary complex at the cell surface. It is postulated that, while in the ternary complex, pro-MMP-2 is fully activated by two sequential cleavages at the pro-domain, which are mediated first by a neighbouring TIMP-2-free MT1-MMP and then, presumably, by a closely associated fully active MMP-2 acting in an intermolecular autocatalytic manner [17]. After activation, the newly activated MMP-2 must dissociate from the ternary complex, since the active enzyme can be detected in the supernatant of cultured cells induced to activate pro-MMP-2 by various agents [30–34], or by overexpression of MT1-MMP [21]. Since gelatin zymography, the most common method to follow pro-MMP-2 activation, cannot assess net proteolytic activity [35], the interplay between MMP-2 and TIMP-2 after activation has been overlooked. The present study was designed to measure the net activity of MMP-2 generated after activation, and to assess whether TIMP-2 affects the level of activity of the released MMP-2. To address this issue, we used a mammalian cell expression system, in which the level of MT1-MMP and TIMP-2 could be controlled by using defined amounts of vaccinia viruses expressing these proteins. We have previously shown that infection of cells with vaccinia viruses minimizes any possible influence of the endogenous MMPs and TIMPs [10], because vaccinia virus significantly inhibits host-protein synthesis. Therefore, the effects of TIMP-2 on MMP-2 activity could be reliably assessed, as opposed to cellular systems expressing natural MMPs and TIMPs or stable/transient transfection systems in which the involvement of endogenous TIMPs cannot be excluded. Furthermore, such cellular systems usually express other MMPs and are thus unsuitable for measurement of MMP enzymic activity because of the lack of MMP-2 specificity. In the present study, we have found that measurable MMP-2 activity was generated in the supernatant of cells expressing MT1-MMP and TIMP-2, and that this activity was modulated by the level of TIMP-2 expression in the system. At the highest levels, TIMP-2 inhibited MMP-2 net proteolytic activity, in spite of the presence of fully active forms, as determined by gelatin zymography. Thus, even at the highest level of TIMP-2, there was considerable cleavage of the pro-domain of pro-MMP-2, and yet MMP-2 activity was undetectable. Furthermore, the increase in pro-MMP-2 activation correlated, to an extent, with the accumulation of active MT1-MMP (57 kDa) on the cell surface and its autocatalytic processing to the inactive membrane-tethered 44-kDa species lacking the catalytic domain [21]. These results demonstrate the complexity of the pro-MMP-2 activation machinery that tightly regulates the proteolytic activity of both MT1-MMP and MMP-2 by TIMP-2. The delicate and dynamic balance between pro-MMP-2, MT1-MMP and TIMP-2 within, as well as outside, the ternary complex determines the amount of active MT1-MMP on the cell surface by controlling its rate of autocatalytic processing and the level of active MMP-2.

In the present study, there was a gradual loss of net MMP-2 activity as a function of TIMP-2 level, which could not be

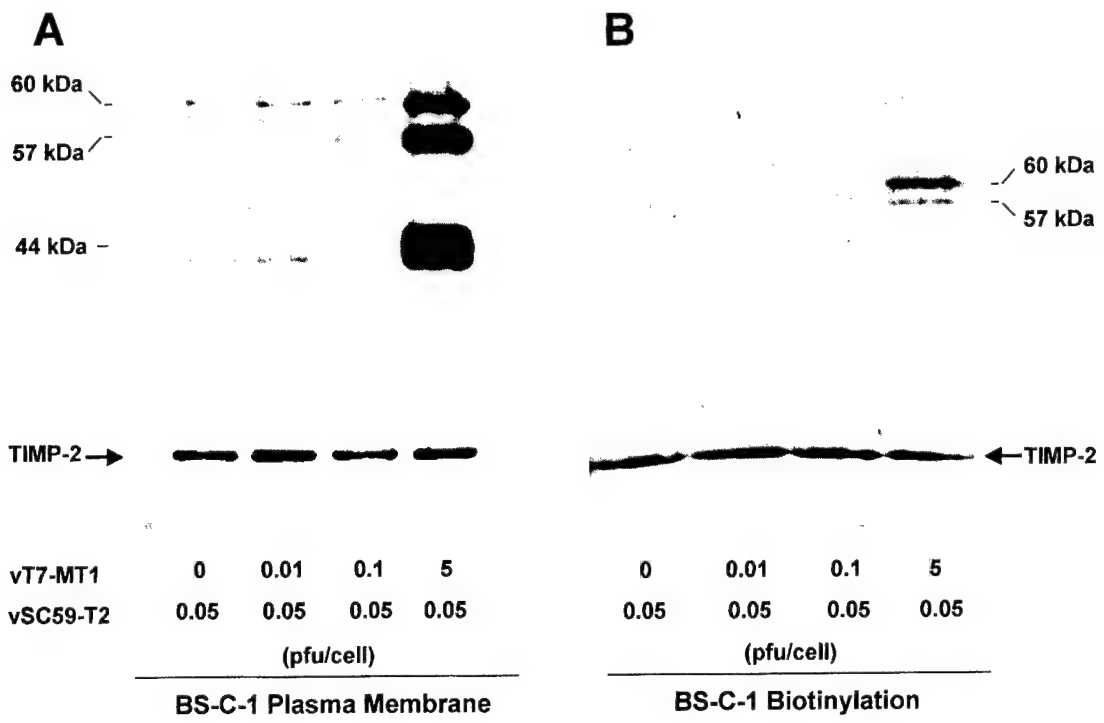

Figure 5 Effect of MT1-MMP expression in TIMP-2 binding to the cell membrane

(**A**) Immunoblot analysis of MT1-MMP and TIMP-2 of PM fractions of BS-C-1 cells infected to express increasing MT1-MMP (indicated by the increasing pfu/cell of vT7-MT1, 0–5 pfu/cell) and constant TIMP-2-expressing vSC59-T2 virus (0.05 pfu/cell) with pAb 437 and mAb CA 101 respectively. (**B**) BS-C-1 cells were co-infected with vTF7-3 (5 pfu/cell), vSC59-T2 (0.05 pfu/cell) and increasing vT7-MT1 virus (0–5 pfu/cell), biotinylated and lysed, as described in the Materials and methods section. The lysates were centrifuged, and the supernatants were incubated with streptavidin–agarose beads. The beads were washed, treated with reducing Laemmli sample buffer and boiled. After centrifugation, the supernatants were subjected to immunoblot analysis with pAb 160 and mAb CA-101, against MT1-MMP_{cat} and TIMP-2 respectively.

identified by zymography, and by the accumulation of active MT1-MMP observed by immunoblot analysis. Inhibition was probably mediated by TIMP-2 binding to the active MMP-2, which was either released from the ternary complex with MMP-2 or secreted by the cells independently of the MT1-MMP/TIMP-2 axis, or both. To address the source of the inhibitory TIMP-2, we used the PM fraction of the infected cells, thus avoiding TIMP-2 secretion. These studies showed a pattern of MMP-2 activity and inhibition analogous to that observed with live cells. Although in the cells, the importance of the secreted TIMP-2 cannot be ignored, the studies with the PM fractions suggest a critical role for cell-surface-associated TIMP-2 in mediating the inhibition of the activated MMP-2. Indeed, our results indicate that MMP-2 can displace PM-associated TIMP-2, resulting in inhibition of enzymic activity. However, the source of the TIMP-2 in the PM that is displaced by MMP-2 is unlikely to be the TIMP-2 bound to MT1-MMP. First, MMP-2 was not efficient in displacing TIMP-2 from a pre-formed complex of the inhibitor with a recombinant MT1-MMP_{cat}. Because TIMP-2 is a slow, tight-binding inhibitor of both MMP-2 and MT1-MMP [22,25], MMP-2 displacement of TIMP-2 from its binding to MT1-MMP_{cat} is not prompt and requires high concentrations of MMP-2 and long incubation periods. Although this process cannot be completely ruled out, as we have shown in the displacement experiments, it is unlikely that active MMP-2 can displace MT1-MMP-bound TIMP-2 under physiological conditions, where the enzyme levels tend to be low. Secondly, whereas TIMP-2 binds to active MT1-MMP with K_d values in the nanomolar range [15,29], the inhibitor may also associate with the PM via sites that are distinct from MT1-MMP. This is suggested by the observation that the levels of TIMP-2 in the PM fractions were independent of the levels of MT1-MMP expression. Furthermore, surface biotinylation of the cells demonstrated surface-associated TIMP-2, regardless of the levels of MT1-MMP. Moreover, MT1-MMP autocatalytic processing, as determined by the presence of the 44 kDa species, could still be observed at the highest level of TIMP-2 expression suggesting that not all the TIMP-2 present in the cells interacts with MT1-MMP. Taken together, these results suggest that TIMP-2, occupying presumably weaker binding sites other than MT1-MMP on the cell surface, may be recruited by the active MMP-2 resulting in inhibition of enzymic activity. This conclusion is in partial agreement with a previous study [18], which showed that cell-surface-activated MMP-2 is inhibited by TIMP-2 bound to the PM of ConA-treated human uterine cervical fibroblasts at sites distinct from MT1-MMP. This study provided evidence for two populations of TIMP-2 binding sites on the membranes, one sensitive (MT1-MMP), and a second one (40–50% of the sites) insensitive to synthetic hydroxamate inhibitors. In agreement with this possibility, two TIMP-2 binding sites with different affinities (K_d values of 15 and 35 nM) have also been detected in a lymphoma cell line [36]. The existence of alternate TIMP-2-binding sites on the cell surface, which can affect MMP-2 activity, may depend on the nature of the cellular background and on the levels of MT1-MMP and TIMP-2 expression. Although the nature of the low-affinity TIMP-2 binding sites remains to be determined, it is known that TIMP-2 can bind to heparin [37,38] and, therefore, may also interact with surface-associated heparan sulphate moieties.

In summary, we have shown that pro-MMP-2 activation on the cell surface yields MMP-2 free and in complex with TIMP-2 in solution. The relative amount of both forms is regulated by the MT1-MMP/TIMP-2 ratio expressed by the cells, and also by

TIMP-2 bound at sites distinct from MT1-MMP. Conversely, the accumulation of MMP-2 in solution may lead to displacement of TIMP-2 from the cell surface, in a self-regulatory mechanism. Taken together, the results of the present study emphasize the crucial role played by TIMP-2 in the regulation of MMP-2 activity, not only on the cell surface, but also in the extracellular environment, which may have important consequences for extracellular matrix remodelling *in vivo*.

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Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes

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Abstract

MMP-9 (gelatinase B) is produced in a latent form (pro-MMP-9) that requires activation to achieve catalytic activity. Previously, we showed that MMP-2 (gelatinase A) is an activator of pro-MMP-9 in solution. However, in cultured cells pro-MMP-9 remains in a latent form even in the presence of MMP-2. Since pro-MMP-2 is activated on the cell surface by MT1-MMP in a process that requires TIMP-2, we investigated the role of the MT1-MMP/MMP-2 axis and TIMPs in mediating pro-MMP-9 activation. Full pro-MMP-9 activation was accomplished via a cascade of zymogen activation initiated by MT1-MMP and mediated by MMP-2 in a process that is tightly regulated by TIMPs. We show that TIMP-2 by regulating pro-MMP-2 activation can also act as a positive regulator of pro-MMP-9 activation. Also, activation of pro-MMP-9 by MMP-2 or MMP-3 was more efficient in the presence of purified plasma membrane fractions than activation in a soluble phase or in live cells, suggesting that concentration of pro-MMP-9 in the pericellular space may favor activation and catalytic competence.

Keywords: Matrix metalloproteinases; Protease; Protease inhibitor; Plasma membrane; Zymogen

Pericellular proteolysis is an essential physiological process that controls the immediate cellular milieu and thus plays a key role in cellular behavior and survival [1]. The members of the matrix metalloproteinase (MMP) family of zinc-dependent endopeptidases are major mediators of pericellular proteolysis by promoting the degradation of extracellular matrix (ECM) components and cell surface-associated proteins. Among the members of the MMP family, the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are known to be key players in many physiological and pathological processes. The gelatinases are secreted in a zymogenic form (pro-MMP) that is activated in the extracellular space and therefore zymogen activation is an important mechanism of regulation of gelatinase activity. Since several major targets of gelatinase activity are located directly on the cell surface [1–4], specific mechanisms were developed by cells to promote the association and activation of the gelatinases on the cell surface. In the case of pro-MMP-2, surface association and activation is mediated by interactions with the membrane type-MMPs (MT-MMPs), a subfamily of MMPs that is tethered to the plasma membrane (PM) via specific membrane-anchoring domains [5–7]. The activation of pro-MMP-2 by MT1-MMP and by MT3-MMP[1] involves the action of the tissue inhibitor of metalloproteinase (TIMP)-2, which forms a complex with active MT1-MMP that serves as a cell surface "receptor" for pro-MMP-2 [8–10]. Bound pro-MMP-2 is then activated by a neighboring TIMP-2-free MT1-MMP [9], which hydrolyzes the Asn^{37}–Leu^{38} peptide bond in the prodomain of pro-MMP-2 [11]. This process generates an inactive MMP-2 intermediate that is subsequently cleaved at the Asn^{80}–Tyr^{81} peptide bond by a fully active MMP-2, in an intermolecular autocatalytic event. This second step of the activation process eventually leads to full pro-MMP-2 activation [11].

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The ability of the MT-MMP/MMP-2 axis to elicit meaningful pericellular proteolysis is strictly dependent on the action of TIMPs. For example, the level of TIMP-2 controls the rate of pro-MMP-2 activation because at low levels relative to MT1-MMP, TIMP-2 promotes activation by generating pro-MMP-2 "receptor," whereas at high levels, TIMP-2 inhibits this process by blocking all active MT1-MMP [8,10,12]. Also, the level of TIMP-2 determines the net proteolytic activity of MMP-2 in the extracellular space [13]. TIMP-1 is a poor inhibitor of MT-MMPs [11] but is an efficient MMP-2 inhibitor [14]. Thus, TIMP-1 can regulate the autocatalytic conversion of the intermediate form of MMP-2 (~64 kDa) into the active species during the second step of the MT1-MMP/MMP-2 activation cascade [11]. TIMP-3 and TIMP-4 will also influence the activation of pro-MMP-2 by inhibiting MT1-MMP and/or the second step owing to their ability to be effective inhibitors of both MT-MMPs and MMP-2 [15,16]. However, we have recently found that TIMP-3 can enhance the activation of pro-MMP-2 by MT3-MMP but not of MT1-MMP[1]. Therefore, TIMPs can exert both positive and negative effects on the MT-MMP/MMP-2 activation cascade at the cell surface.

Pro-MMP-9 is also associated with surface proteolysis, a process that has profound biological influences [2,3,17,18]. However, as opposed to pro-MMP-2, an MT-MMP/TIMP-dependent mechanism of pro-MMP-9 surface association and activation has not been described. Instead, pro-MMP-9 associates with the cell surface using different mechanisms [3,19–22] and is activated by enzymes from various protease families including other MMPs [23–27]. Among members of the MMP family, MMP-3 [28,29], MMP-2 [30], MMP-13 [31], MMP-7 [32], and MMP-26 [33] were shown to activate pro-MMP-9, albeit with different efficiencies, as determined with purified enzymes. Although distinct proteolytic pathways can lead to activation of pro-MMP-9, many studies examining the role of MMP-9 in cellular systems failed to produce evidence of active enzyme (determined as the presence of a ~82-kDa species), even when a particular cellular activity was ascribed to the action of MMP-9 and known pro-MMP-9 activators were detected in the cells. Thus, the lack of active MMP-9 in culture conditions remains a major paradox in our understanding MMP-9 regulation.

The activation of pro-MMP-9 by MMP-2 is of interest because these enzymes are generally co-expressed in many human cancers and their expression has been correlated with tumor progression. In coordination with MT-MMPs, the gelatinases can accomplish the degradation of multiple pericellular substrates including the interstitial collagen matrix. Using purified enzymes, we previously reported that MMP-2 activates pro-MMP-9 by cleaving at the Glu^{40}–Met^{41} amide bond of the prodomain to generate an 86-kDa form followed by cleavage at the Arg^{87}–Phe^{88} amide bond generating the fully active 82-kDa species in a process similar to the activation of pro-MMP-9 by MMP-3 [30,34]. As mentioned above, the activation of pro-MMP-2 by MT1-MMP is tightly regulated by TIMP-2, which subsequently may affect the activation of pro-MMP-9 by MMP-2. Although MMP-2 can activate pro-MMP-9 in solution, the relationship between the MT1-MMP/MMP-2 axis, TIMPs, and pro-MMP-9 activation on the PM has not been studied. Furthermore, little is known about the activation of pro-MMP-9 by MMP-3 on the PM. Here, we investigated the roles of MMPs and TIMPs in regulating the activation of pro-MMP-9 using purified PM of cells expressing distinct levels of enzymes and inhibitors. We show that pro-MMP-9 activation is a highly regulated surface-associated process that is accomplished only under restricted conditions and facilitated by the PM environment.

Materials and methods

Cell culture. Non-malignant monkey kidney epithelial BS-C-1 cells (CCL-26) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. HeLa cells (CCL-2.2) were cultured in Spinner medium (Quality Biologicals, Gaithersburg, MD) supplemented with 5% horse serum and antibiotics. Non-malignant human breast epithelial MCF10A cells were cultured as described [22].

Recombinant vaccinia viruses. The production of the recombinant vaccinia virus (vTF7-3) expressing bacteriophage T7 RNA polymerase has been described [35]. Recombinant vaccinia viruses expressing either human TIMP-2 (vSC59-T2) or MT1-MMP (vTF-MT1) were obtained by homologous recombination as previously described [35].

Recombinant proteins and antibodies. Human recombinant pro-MMP-2, pro-MMP-9, TIMP-2, and TIMP-1 were expressed in HeLa cells (CCL-2.2) infected with the appropriate recombinant vaccinia viruses and purified to homogeneity, as previously described [14]. A truncated pro-MMP-9 (Met^{1}–Thr^{498}) lacking the hemopexin-like domain (pro-ΔHLD-MMP-9) was constructed by inserting a stop codon after Thr^{498} by site-directed mutagenesis in the full-length cDNA of human pro-MMP-9 cloned into the pTF7-EMCV-1 expression vector [35]. After DNA sequence verification, the truncated enzyme was expressed in BS-C-1 cells using the infection-transfection procedure, as described [16,36]. Twenty-four hours later, the media were collected and the truncated pro-MMP-9 was purified by gelatin affinity chromatography, as previously described [37]. The catalytic domain of human recombinant MT1-MMP (MT1-MMP_{cat}) was purchased from Calbiochem (San Diego, CA). The anti-TIMP-2 monoclonal antibody (mAb) CA-101 [36], the rabbit polyclonal antibody (pAb) against the catalytic domain of MMP-9, pAb 110 [38], and the rabbit pAb 437 and pAb 198 to the hemopexin-like domain and catalytic domain of MT1-MMP [10], respectively, have been described previously.

Co-expression of MT1-MMP and TIMP-2 and isolation of PM. Confluent cultures of BS-C-1 cells in 150-mm dishes were infected with 5 plaque-forming units (pfu)/cell each of vTF7-3 and vT7-MT1 viruses to express MT1-MMP alone or co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 viruses and different amounts (0.01, 0.1 or 1 pfu/cell) of the TIMP-2-expressing virus vSC59-T2, to express MT1-MMP with increasing levels of TIMP-2 [10]. As a control, the cells were infected only with the vTF7-3 virus. After infection, the virus-containing media were aspirated and replaced with serum-free DMEM followed

by overnight incubation. Then, the media were aspirated and the cells were subjected to subcellular fractionation to isolate the PM fraction, as previously described [10]. The purified PM fractions were collected and the protein concentration was determined by the BCA procedure (Pierce, Rockford, IL). The PM fractions were stored at −80 °C until used in zymogen activation studies.

Activation of pro-MMP-2 and pro-MMP-9 in cells expressing MT1-MMP. Confluent cultures of BS-C-1 cells in 6-well plates were infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 viruses for 45 min in infection media (DMEM supplemented with 2.5% FBS and antibiotics) at 37 °C. After infection, the cells were incubated (16 h, 37 °C) with serum-free DMEM supplemented with human recombinant TIMP-2 (0, 1 or 10 nM). The media were aspirated and the cells were rinsed with serum-free DMEM followed by incubation with fresh serum-free media supplemented with pro-MMP-2 and/or pro-MMP-9 (10 nM each) for an additional 16 h at 37 °C. The supernatants were collected and subjected to gelatin zymography for analysis of pro-MMP-2 and pro-MMP-9 activation, as previously described [38].

Activation of pro-MMP-2 and pro-MMP-9 by soluble MT1-MMP$_{cat}$ or by PM fractions. Purified human MT1-MMP$_{cat}$ (50 nM) was incubated (16 h, 37 °C) with pro-MMP-2 or pro-MMP-9 (50 nM final concentration each) in collagenase buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35). In some experiments, pro-MMP-2 and pro-MMP-9 (50 nM each) were incubated with 25 nM MT1-MMP$_{cat}$ in the absence or presence of 10 or 50 nM TIMP-2 or TIMP-1. Equal amounts of purified PM fractions (3.5 μg per 50 μl reaction) were incubated (16 h, 37 °C) with either pro-MMP-2 (5 or 50 nM) or pro-MMP-9 (50 nM) alone or a mixture of pro-MMP-2 and pro-MMP-9 in the absence or presence of TIMP-1 (various molar ratios relative to pro-MMP-9). Pro-gelatinase activation was monitored by gelatin zymography, as described [38].

Ultracentrifugation of PMs. PMs (3.5 μg per 50 μl reaction) of BS-C-1 cells expressing MT1-MMP alone or MT1-MMP and increasing amounts of TIMP-2 were incubated (16 h, 37 °C) with 10 nM pro-MMP-2 to generate active MMP-2. Then, half of the reaction mixture was subjected to ultracentrifugation (1 h, 100,000*g*) and the other half was kept on ice. After the ultracentrifugation, the resultant supernatant (soluble fraction) and pellet (membrane-associated fraction) fractions were collected and the latter was resuspended in collagenase buffer. Then, each of the three fractions (non-centrifuged fraction, soluble, and membrane-associated) was incubated (16 h, 37 °C) with pro-MMP-9 (50 nM). Aliquots of the reaction mixtures were analyzed by gelatin zymography and by immunoblot analysis as described [38].

MMP-9 fluorescent activity assay. PMs were isolated from BS-C-1 cells co-infected with 5 pfu/cell each of vTF7-3 and vT7-MT1 viruses to express MT1-MMP alone or with 5 pfu/cell each of vTF7-3 and vT7-MT1 viruses and 0.01 pfu/cell of vSC59-T2 to co-express MT1-MMP and TIMP-2. The PM fractions were incubated (16 h, 37 °C) with either 50 nM pro-MMP-9 or with pro-MMP-9 and pro-MMP-2 (50 nM each) in 50 μl of collagenase buffer. As a control, pro-MMP-9 or pro-MMP-9 and pro-MMP-2 (50 nM each) were incubated without PMs under the same conditions. At the end of the incubation period, the samples were diluted (1000-fold) and the MMP-9 activity was measured using the Fluorokine E, human active MMP-9 Fluorescent assay (R&D System, Minneapolis, MN) according to the manufacturer's instruction. The amount of active MMP-9 in each sample was determined using a standard curve of APMA-activated pro-MMP-9.

Pro-MMP-9 activation by MMP-3 in PMs of MCF10A cells. PMs were isolated from breast epithelial MCF10A cells, as previously described [38]. Human recombinant pro-MMP-3 was heat activated at 55 °C for 1 h and the amount of catalytically competent MMP-3 was determined by active-site titration with human recombinant TIMP-1, as described [39]. Pro-MMP-9 (96 nM) was incubated with MMP-3 (1 nM) and increasing amounts of MCF10A PMs (0–93 μg/ml), in collagenase buffer without Brij 35, at 37 °C. At various times (up to 2 h), aliquots of the reaction mixture (20 μl) were removed for MMP-9 assay with the synthetic fluorogenic substrate MOCAcPL-GLA$_2$pr(Dnp)AR-NH$_2$ (Peptides International, Louisville, KY), as previously described [39]. Less than 10% of hydrolysis of the fluorogenic substrate was monitored as described by Knight [40]. Hydrolysis of this peptide by MMP-3 was insignificant relative to MMP-9, under the experimental conditions used. The MMP-9 concentration was determined using the Michaelis–Menten equation, and the k_{cat} and K_m values for the reaction of MMP-9 with the synthetic peptide substrate ($4.41 \pm 0.55\ s^{-1}$ and $2.46 \pm 0.34\ \mu M$, respectively [14]. Initial velocities of pro-MMP-9 activation were measured from the linear increase in MMP-9 concentration as a function of time.

Results

MT1-MMP/MMP-2-dependent activation of pro-MMP-9 in solution and in live cells

We first examined the role of MT1-MMP and MMP-2 in activation of pro-MMP-9 using purified enzymes. As described previously [11], a soluble catalytic domain of MT1-MMP (MT1-MMP$_{cat}$) can initiate the activation of pro-MMP-2 in the absence of TIMP-2 leading to full activation (Fig. 1A). In contrast, MT1-MMP$_{cat}$

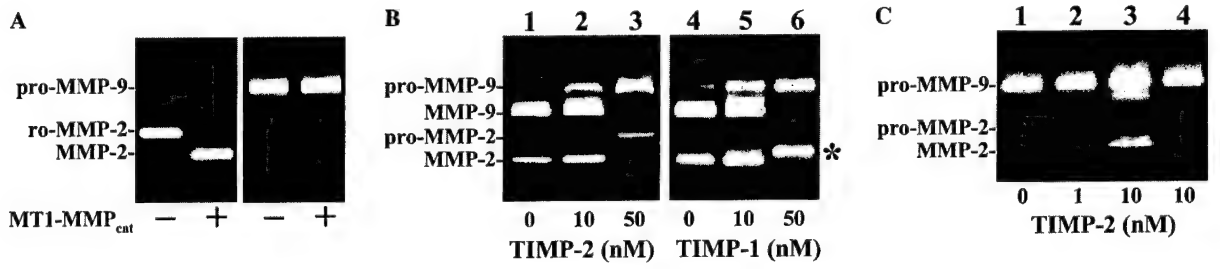

Fig. 1. Activation of pro-MMP-9 by MMP-2 in solution and in BS-C-1 cells. (A) Pro-MMP-2 and pro-MMP-9 (50 nM each) were incubated (16 h, 37 °C) without (−) or with (+) MT1-MMP$_{cat}$ (50 nM) in collagenase buffer. Aliquots of the reaction mixtures were collected and analyzed by gelatin zymography. (B) Pro-MMP-2 (50 nM), pro-MMP-9 (50 nM), and MT1-MMP$_{cat}$ (25 nM) were incubated (16 h, 37 °C) without or with TIMP-2 or TIMP-1 (10 or 50 nM) in collagenase buffer. Aliquots of the reaction mixtures were collected and analyzed by gelatin zymography. The asterisk indicates the intermediate form of MMP-2 (~66 kDa). (C) BS-C-1 cells were infected to express MT1-MMP as described in Materials and methods and then incubated (16 h, 37 °C) with recombinant TIMP-2 (0, 1 or 10 nM). The media were aspirated and the cells were incubated with serum-free media supplemented with pro-MMP-2 and/or pro-MMP-9 (10 nM each) for an additional 16 h at 37 °C. Gelatinase activation was monitored by gelatin zymography.

cannot activate pro-MMP-9 (Fig. 1A). However, pro-MMP-9 is almost fully activated in the presence of pro-MMP-2 and MT1-MMP_{cat}, as a result of generation of active MMP-2 (Fig. 1B, lanes 1 and 4). Addition of 50 nM TIMP-2 to the reaction mixture strongly inhibits both pro-MMP-2 and pro-MMP-9 activation (Fig. 1B, lane 3) by inhibiting MT1-MMP_{cat}. Addition of TIMP-1 (50 nM) results in the accumulation of the intermediate form of MMP-2 and consequently pro-MMP-9 activation is inhibited (Fig. 1B, lane 6). TIMP-1 is a weak inhibitor of MT1-MMP but inhibits the second step of pro-MMP-2 activation by inhibiting MMP-2 [11].

To study the effects of the MT1-MMP/MMP-2 axis on pro-MMP-9 activation in live cells, we expressed MT1-MMP in BS-C-1 cells, as we have previously described [10]. The MT1-MMP-expressing cells were incubated (16 h) without or with exogenous TIMP-2 (1 or 10 nM), washed to remove excess inhibitor, and then incubated (16 h) with a mixture of pro-MMP-2 and pro-MMP-9 (10 nM each). Gelatinase activation in the supernatant was monitored by gelatin zymography. As shown in Fig. 1C, addition of 10 nM TIMP-2 induced significant pro-MMP-2 activation (Fig. 1C, lane 3), as expected. In the absence of TIMP-2 (Fig. 1C, lane 1), some activation of pro-MMP-2 was observed due to the long incubation time and the presence of a small amount of endogenous TIMP-2 produced by the BS-C-1 cells, as we previously reported [41]. In the presence of active MMP-2, pro-MMP-9 was only processed to the intermediate form of ~85 kDa (Fig. 1C, lane 3). This processing was specific since in the absence of TIMP-2 (Fig. 1C, lane 1) or MMP-2 (Fig. 1C, lane 4), pro-MMP-9 processing was not observed. Thus, whereas full activation of pro-MMP-9 could be achieved by MMP-2 in a purified system, only limited pro-MMP-9 processing (to the intermediate form) was observed in a cellular system engineered to express MT1-MMP. Taken together, these studies suggest that pro-MMP-9 is activated by MMP-2 in a cascade of zymogen activation initiated by MT1-MMP, which is regulated by TIMP-2 and TIMP-1 at various steps of the activation cascade.

MT1-MMP/MMP-2-dependent activation of pro-MMP-9 with PM

Since full pro-MMP-9 activation was not observed in the live cells, under these conditions, we speculated that interaction of the MT1-MMP/MMP-2 axis with pro-MMP-9 on the PM would generate a microenvironment conducive to full activation. Therefore, we isolated the PM fractions of BS-C-1 cells that were infected to express constant levels of MT1-MMP and increasing amounts of TIMP-2, thus generating various ratios of MT1-MMP and TIMP-2 on the cell surface. The isolated PM fractions were characterized for the presence of MT1-MMP and TIMP-2 by immunoblot analysis. As shown in Figs. 2A and B, MT1-MMP was detected as a 60-kDa form representing the latent form and a 44-kDa inactive form representing an autocatalytic degradation product lacking the catalytic domain [10]. This profile of MT1-MMP forms was dependent on the level of TIMP-2, as we described earlier [10]. Increasing amounts of TIMP-2 in the PM fractions (Fig. 2C) correlated with accumulation of mature MT1-MMP (57 kDa) and reduction of the 44-kDa degradation product (Figs. 2A and B), consistent with the ability of TIMP-2 to inhibit the autocatalytic processing of MT1-MMP [10]. Next, we tested the ability of the PM fractions to activate two different concentrations of pro-MMP-2 (5 and 50 nM). These studies showed that the degree of activation was dependent on the amount of exogenous pro-MMP-2 added to the reaction (Figs. 2D and E). At 50 nM, sustained pro-MMP-2 activation was observed regardless of the level of TIMP-2 in the PM fraction (Fig. 2D), indicating that TIMP-2 was not limiting the activation process. On the other hand, a lower pro-MMP-2 concentration (5 nM) produced a pattern of activation that was dependent on the level of TIMP-2 (Fig. 2E, lanes 3–6) with more mature MMP-2 at the lowest levels of inhibitor and inhibition of activation at the highest level of TIMP-2. Because the sustained activation of pro-MMP-2 was observed at the

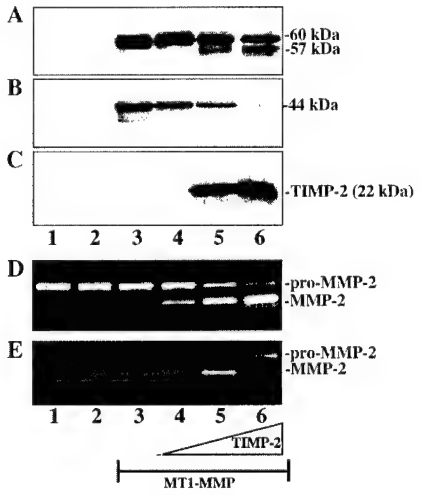

Fig. 2. Activation of pro-MMP-2 by MT1-MMP in isolated PMs of BS-C1-cells. (A–C) Immunoblot analysis of MT1-MMP (A–B) and TIMP-2 (C) in isolated PM fractions of BS-C-1 cells. PM of non-infected cells (lane 1), PM of control infected cells (lane 2), and PM of cells infected to express MT1-MP alone (lane 3) or constant levels of MT1-MMP with increasing amounts of TIMP-2 (lanes 4–6) were subjected to reducing 12% SDS–PAGE followed by immunoblot analysis using pAb 198 (A) and pAb 437 (B) to MT1-MMP, and mAb 101 (C) to TIMP-2. The molecular mass (kDa) in (A) and (B) indicates the relative mass of the MT1-MMP forms. (D,E) Gelatin zymography of pro-MMP-2 activation by PM fractions. Five (D) or 50 (E) nM of pro-MMP-2 was incubated (16 h, 37 °C) with PM fractions (3.5 μg per 50 μl reaction) of BS-C-1 cells as described in (A–C). Aliquots of the reaction mixtures were analyzed by gelatin zymography.

50 nM concentration, this amount of pro-MMP-2 was chosen to carry out the experiments with pro-MMP-9.

The PM fractions were tested for their ability to initiate a cascade of zymogen activation leading to the activation of pro-MMP-2 and pro-MMP-9. As shown in Fig. 3A, pro-MMP-9 was not activated when incubated with the PM fractions in the absence of pro-MMP-2. Addition of a mixture of pro-MMP-2 and pro-MMP-9 (50 nM each) to the PM fractions resulted in the processing of pro-MMP-9 to the ~82-kDa active species (Fig. 3B, lanes 4–6) in all the samples containing active MMP-2. A fluorometric activity assay specific for MMP-9 confirmed that the 82-kDa generated by MMP-2 in the presence of TIMP-2 and PMs was a fully active enzyme (Fig. 4, lane 4). Based on the amount of pro-MMP-9 present in the reactions and a standard curve of active MMP-9, we determined that 61% of pro-MMP-9 was activated by MMP-2 under these conditions. The negative controls (Fig. 4, lanes 1, 2, 3, and 5) showed background levels of MMP-9 activity including PMs with MT1-MMP alone (Fig. 5, lane 5) demonstrating the importance of TIMP-2 for pro-MMP-2 activation and consequently in the activation of pro-MMP-9. Thus, the activation of pro-MMP-9 by the MT1-MMP/MMP-2 axis in the presence of TIMP-2 generates fully active MMP-9.

The hemopexin-like domain of pro-MMP-9 is not required for activation of pro-MMP-9 MMP-2

The hemopexin-like domain of MMPs has been shown to be involved in protein–protein interactions

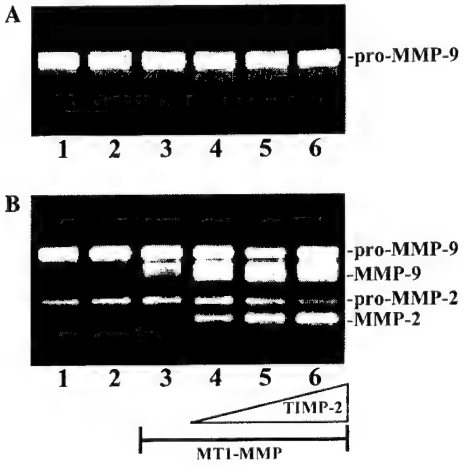

Fig. 3. Activation of pro-MMP-9 and pro-MMP-2 in PMs. Pro-MMP-9 (50 nM) was incubated (16 h, 37 °C) with equal amounts (3.5 μg each) of PM fractions of non-infected cells (lane 1), PM of control infected cells (lane 2), PM of cells infected to express MT1-MP alone (lane 3) or PM from cells co-infected to express constant levels of MT1-MMP with increasing amounts of TIMP-2 (lanes 4–6), in the absence (A) or presence (B) of 50 nM pro-MMP-2 in 50 μl of collagenase buffer. Aliquots of the reaction mixtures were analyzed by gelatin zymography.

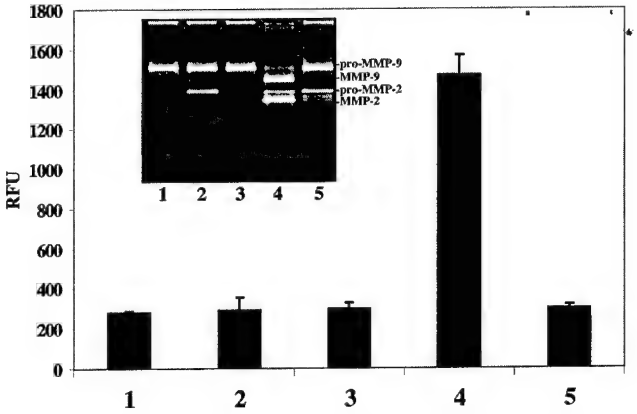

Fig. 4. Activation of pro-MMP-9 by MMP-2 in PM fractions containing MT1-MMP and TIMP-2 generates MMP-9 enzymatic activity. Samples were prepared as described in Materials and methods. Lane, 1, pro-MMP-9 alone; lane 2, pro-MMP-9 + pro-MMP-2; lane 3, pro-MMP-9 + PM containing MT1-MMP and TIMP-2; lane 4, pro-MMP-9 and pro-MMP-2 + PM containing MT1-MMP and TIMP-2, and lane 5, pro-MMP-9 and pro-MMP-2 + PM containing MT1-MMP alone. The samples were analyzed by gelatin zymography (insert) and by the Fluorokine E, human active MMP-9 Fluorescent assay according to the manufacturer's instruction. RFU, relative fluorescence unit.

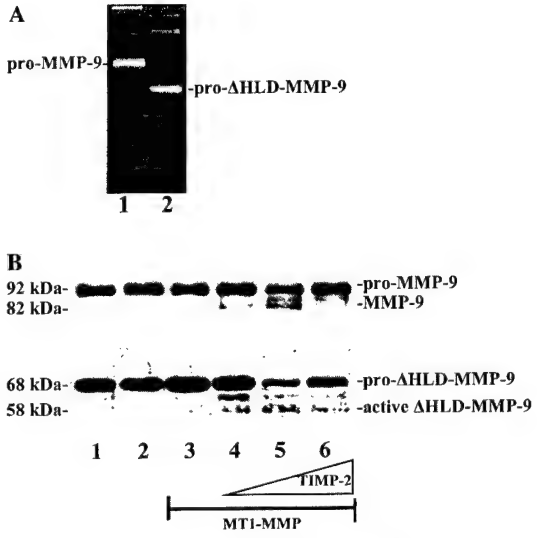

Fig. 5. The hemopexin-like domain of pro-MMP-9 is not required for MMP-2 activation in PM fractions. (A) Equal amounts (10 ng) of wild-type pro-MMP-9 (lane 1) and pro-ΔHLD-MMP-9 (lane 2) were analyzed by gelatin zymography. (B) Wild-type pro-MMP-9 (upper panel) and pro-ΔHLD-MMP-9 (lower panel) (50 nM each) were incubated (16 h, 37 °C) with either PM of non-infected cells (lane 1), PM of control infected cells (lane 2), PM of cells infected to express MT1-MP alone (lane 3) or PM of cells co-infected to express constant levels of MT1-MMP with increasing amounts of TIMP-2 (lanes 4–6) in the presence of pro-MMP-2 (50 nM). Aliquots of the reaction mixtures were subjected to reducing 10% (upper panel) or 12% (lower panel) SDS-PAGE followed by immunoblot analysis using the rabbit pAB 110 to the catalytic domain of pro-MMP-9. The molecular mass (kDa) represents the relative mass of the MMP-9 forms.

and in the case of pro-MMP-2 in surface activation [42]. Thus, we examined whether the hemopexin-like domain of pro-MMP-9 was required for activation of pro-MMP-9 by the MT1-MMP/MMP-2 axis in the PM fractions. To this end, we generated a truncated pro-MMP-9 form lacking the entire hemopexin-like domain (pro-ΔHLD-MMP-9). As shown in Fig. 5A, the truncated enzyme exhibited gelatinolytic activity in zymograms that were equivalent to that exhibited by the wild-type pro-MMP-9. The PM fractions were incubated with either wild-type pro-MMP-9 or pro-ΔHLD-MMP-9 in the presence of pro-MMP-2 and the proteolytic cleavage was detected by immunoblot analysis using the pAb 110 directed to the catalytic domain of MMP-9. This method was chosen to assess activation because in zymograms pro-ΔHLD-MMP-9 (68 kDa) and its active species (58 kDa) showed an electrophoretic mobility similar to that of MMP-2 (62 kDa). As shown in Fig. 5B, pro-ΔHLD-MMP-9 was processed to a 58-kDa species consistent with removal of the prodomain via generation of an intermediate form (Fig. 5B, lower panel) in a process similar to that exhibited by the wild-type enzyme (Fig. 5B, upper panel). The truncated pro-MMP-9 was also processed by MMP-3 in solution like the wild-type pro-MMP-9 (data not shown).

TIMP-1 regulates pro-MMP-9 activation by MMP-2 in the PM fractions

TIMP-1 binds to pro-MMP-9 and thus can regulate zymogen activation [34]. Therefore, we examined the activation of pro-MMP-9 by the MT1-MMP/MMP-2 axis in the PM fractions in the presence of TIMP-1. To this end, the PM fractions were incubated with various molar ratios of pro-MMP-2 and pro-MMP-9 (1:1 and 1:10; Figs. 6A and C and Figs. 6B and D, respectively) in the absence or presence of either a 2-fold or a 5-fold excess TIMP-1 over pro-MMP-9. As shown in Figs. 6A and B, MMP-2 activated pro-MMP-9 both at 1:1 and at 1:10 pro-MMP-2:pro-MMP-9 molar ratios. A 2-fold excess TIMP-1 over pro-MMP-9 significantly inhibited its activation (Fig. 6C, lanes 4 and 5) when compared to the activation without inhibitor (Fig. 6A, lanes 4 and 5). However, this concentration of TIMP-1 had little effect on pro-MMP-2 activation (Fig. 6C), with the exception that at the highest level of TIMP-2, appearance of the intermediate form of MMP-2 (~66 kDa) was detected (Fig. 6C, lane 6, asterisk). Thus, under these conditions, inhibition of pro-MMP-9 activation was likely to be due to inhibition of the already active MMP-2. A 5-fold excess TIMP-1 over pro-MMP-9 almost completely inhibited pro-MMP-9 activation and also significantly reduced pro-MMP-2 activation (Fig. 6D), as indicated by the accumulation of the intermediate MMP-2 form (Fig. 6D, lanes 4–6) [11].

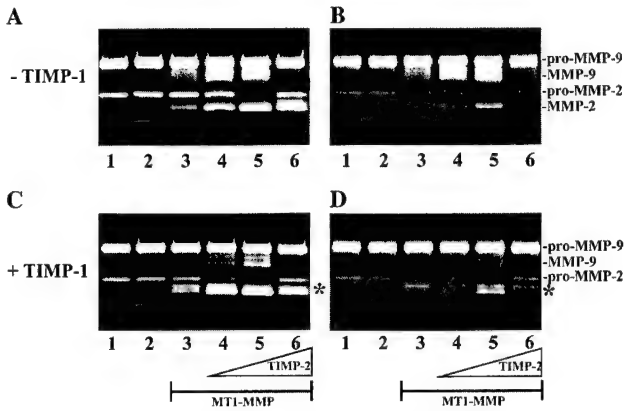

Fig. 6. Effect of TIMP-1 on pro-MMP-9 activation. PM of non-infected cells (lane 1), PM of control infected cells (lane 2), PM of cells infected to express MT1-MP alone (lane 3) or PM of cells co-infected to express constant levels of MT1-MMP with increasing amounts of TIMP-2 (lanes 4–6) was incubated (16 h, 37 °C) with a 1:1 (A,C) or a 1:10 (B,D) molar ratio of pro-MMP-2:pro-MMP-9 in the absence (A,B) or presence (C,D) of TIMP-1 (C, 1:1:2 pro-MMP-2:pro-MMP-9:TIMP-1 and D, 1:10:50 pro-MMP-2:pro-MMP-9:TIMP-1 molar ratios). Aliquots of the reaction mixtures were analyzed by gelatin zymography. The asterisk indicates the intermediate form of MMP-2 (~66 kDa).

The PM facilitates pro-MMP-9 activation

To determine the relative contribution of the PM surface to the activation of pro-MMP-9 by MMP-2, the PM fractions were subjected to ultracentrifugation after pro-MMP-2 activation, as described in Materials and methods. The resultant supernatant and pellet fractions were compared with the whole PM fraction without ultracentrifugation for their ability to activate pro-MMP-9. As shown in Fig. 7B, the supernatant fraction

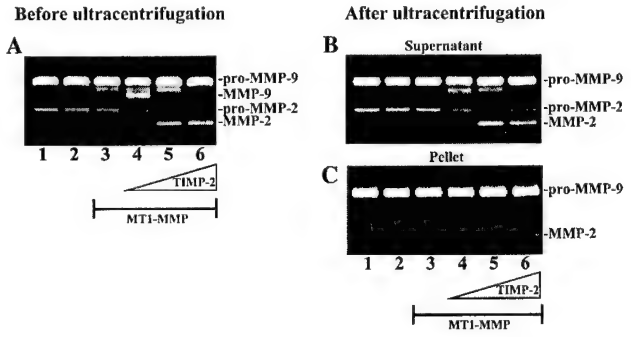

Fig. 7. Ultracentrifugation of PM fractions and effect on pro-MMP-9 activation. PM of non-infected cells (lane 1), PM of control infected cells (lane 2), PM of cells infected to express MT1-MP alone (lane 3) or PM of cells co-infected to express constant levels of MT1-MMP with increasing amounts of TIMP-2 (lanes 4–6) was incubated (16 h, 37 °C) with pro-MMP-2 (10 nM) to generate active MMP-2. One half of each sample was incubated (16 h, 37 °C) with pro-MMP-9 (50 nM) and analyzed by gelatin zymography (A). The other half of the samples was subjected to ultracentrifugation (B,C). The resultant supernatant (B) and pellet (C) of each sample were collected and incubated (16 h, 37 °C) with pro-MMP-9 (50 nM) followed by analysis by gelatin zymography.

exhibited both latent and active soluble MMP-2, indicating that these enzyme forms were not retained in the PM after activation by MT1-MMP. This fraction only caused a partial processing of pro-MMP-9 to the intermediate form (Fig. 7B, lane 4). Interestingly, the pellet fraction, which showed traces of bound active MMP-2, had no effect on pro-MMP-9 activation (Fig. 7C, lanes 4–6). This is likely to be due to the dissociation of MMP-2 from the PM into the supernatant after activation by MT1-MMP, in agreement with a previous study [13]. Pro-MMP-9 activation was readily detected after incubation with the PM fractions that were not subjected to ultracentrifugation (Fig. 7A, lane 4) and thus containing both the insoluble and soluble components of the activation complex. This suggests that the activation of pro-MMP-9 by MMP-2 is enhanced by the presence of the PM surface.

Since the PM surface had a positive effect on activation of pro-MMP-9 by MMP-2, we examined whether the PM also influenced the activation of pro-MMP-9 by MMP-3, a known pro-MMP-9 activator [28]. We chose MMP-3 because of the possibility of obtaining quantitative data using a fluorogenic peptide substrate that is cleaved by MMP-9 but it is a poor substrate for MMP-3 [43]. The PM fraction was isolated from human breast epithelial MCF10A cells, which we showed previously to bind pro-MMP-9 with high-affinity [21]. These cells express low levels of endogenous pro-MMP-9 and no detectable pro-MMP-2 [38]. As depicted in Fig. 8, the initial rates of pro-MMP-9 activation by MMP-3 increased ~3-fold as the PM protein amount was varied between 0 and 5.6 μg, decreasing slightly as the PM concentration was increased further. At the concentrations used, the PM showed no effect on MMP-3 activity (data not shown). Thus, pro-MMP-9 activation by MMP-3 was enhanced in the presence of PM from MCF10A cells.

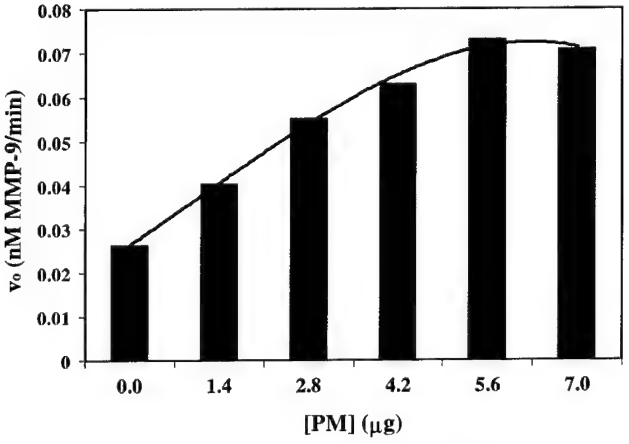

Fig. 8. The PM of MCF10A cells enhances pro-MMP-9 activation by MMP-3. Pro-MMP-9 (96 nM) was incubated with MMP-3 (1 nM) and varying amounts of PM fraction from MCF10A cells at 37 °C. At various times aliquots of the reaction mixture (20 μl) were assayed with the fluorogenic substrate MOCAcPLGLA$_2$pr(Dnp)AR-NH$_2$ as described in Materials and methods.

Discussion

The control of pericellular proteolysis is the result of a delicate balance between proteases and their inhibitors. In general, the main function of protease inhibitors is to terminate proteolysis, thus avoiding excessive damage to the cellular proteosome. However, this view has been challenged by the complex role that TIMP-2 plays in pro-MMP-2 activation, in which the inhibitor is required for the generation of the active enzyme by MT1-MMP via the formation of a ternary complex [6,7]. TIMP-2 can also reduce the autocatalytic turnover of MT1-MMP on the cell surface further contributing to pericellular proteolysis [41,44]. Here, we show that in addition to these effects, TIMP-2 can indirectly promote the activation of pro-MMP-9 by its ability to control pro-MMP-2 activation. Thus, TIMP-2 regulates the cascade of zymogen activation initiated by MT1-MMP that culminates with the generation of two powerful gelatinases, MMP-2 and MMP-9, on the cell surface. The activation of pro-MMP-9 by MMP-2 generated an ~82 kDa form, as determined by gelatin zymography. However, assessment of pro-MMP-9 activation by gelatin zymography alone is limited by the ability of this technique to clearly resolve the fully active MMP-9 species from the intermediate inactive form, when using supernatants, or from the underglycosylated pro-MMP-9 precursor, when using cell lysates [38,45]. Thus, using an activity assay that specifically measures MMP-9 activity, we showed that the activation process results in the generation of catalytically active MMP-9. The activation of pro-MMP-9 by the PM fractions required active MMP-2, since PM fractions alone were unable to process pro-MMP-9. Consistently, a purified catalytic domain of MT1-MMP was also incapable of activating pro-MMP-9. This is not unique to MT1-MMP, since the catalytic domains of MT2-, MT3-, and MT4-MMP were also ineffective (data not shown) suggesting that most members of the MT-MMP subfamily are not direct activators of pro-MMP-9, at least in solution. Although pro-MMP-9 forms a complex with TIMP-1, a mechanism of cellular activation of pro-MMP-9 mediated by MT-MMPs analogous to that described for the pro-MMP-2/TIMP-2 complex has not been described. However, such process is unlikely to exist for pro-MMP-9 since the type I transmembrane MT-MMPs (MT1-, MT2-, MT3-, and MT45-MMP) exhibit low affinity for TIMP-1 [7]. In contrast, the glycophosphatidylinositol (GPI)-anchored MT4- and MT6-MMPs are efficiently inhibited by TIMP-1 [46] but their role in pro-MMP-9

activation' remains unclear. In solution, a catalytic domain of MT4-MMP failed to activate pro-MMP-9 (data not shown) and conflicting results have been reported in regards to the ability of MT6-MMP to activate pro-MMP-9 [46,47]. This evidence suggests that pro-MMP-9 activation in cells is inherently different from that exhibited by its homologue pro-MMP-2, in which surface association and complex with TIMP-2 via the hemopexin-like domain of the zymogen is an important aspect of the activation process. On the other hand, our studies suggest that the hemopexin-like domain of pro-MMP-9 is not necessary for the activation of pro-MMP-9 by MMP-2 in the PM fractions.

We showed that the activation of pro-MMP-9 by the MT1-MMP/MMP-2 axis is tightly regulated by the levels of TIMPs and by the concentration of pro-MMP-9 and MMP-2. While MMP-2 is an efficient activator of pro-MMP-9 in solution [30], we have consistently found that in different cellular systems, pro-MMP-9 activation is generally absent even in the presence of active MMP-2. For example, cultured human HT1080 fibrosarcoma cells, which express both pro-MMP-2 and pro-MMP-9, do not activate pro-MMP-9 even after treatments that induce pro-MMP-2 activation such as phorbol ester [48,49], concanavalin A [49], fibronectin [44], and collagen IV [50]. Several possibilities may explain this deficiency including high levels of TIMPs, lack of reduced levels of activators, and/or the inability of pro-MMP-9 to interact with its potential activators in the surface and/or the media. Previous studies have shown that the molar ratio of pro-MMP-9 to TIMP-1 dictates whether pro-MMP-9 can be activated by MMP-3 in a breast cancer cell line overexpressing pro-MMP-9 [29]. In agreement with these studies, our data show that both TIMP-2 and TIMP-1 can dictate whether MMP-2 will activate pro-MMP-9 by influencing the activation cascade at various steps. For example, at low concentrations relative to MT1-MMP, TIMP-2 enhances pro-MMP-2 activation and consequently the activation of pro-MMP-9. In contrast, a high concentration of TIMP-2 inhibits both MT1-MMP and MMP-2 and the activation of pro-MMP-9 is inhibited. We have also found that the presence of mature MMP-2 (62 kDa), as determined by zymography, does not necessarily correlate with pro-MMP-9 activation. This paradoxical effect is due to the inhibition of MMP-2, which upon release from the ternary complex after activation is inhibited by TIMP-2, as we have recently shown [13]. The inhibitory effect of TIMP-1 on pro-MMP-9 activation is related to its ability to block the autocatalytic processing of the intermediate inactive form of MMP-2 to the fully active form via inhibition of active MMP-2, as previously described [11]. TIMP-1 can also form a non-covalent complex with pro-MMP-9, which may inhibit activation [34]. Thus, a delicate balance between TIMPs and MMPs exerts a tight control over the process of pro-MMP-9 activation in cultured cells by the MT1-MMP/MMP-2 axis. Considering all these factors, there is an inherent difficulty in cellular settings to achieve the right window of natural conditions that lead to full pro-MMP-9 activation.

The expression and/or levels of pro-MMP-9 activators in the cells are also critical to achieve activation. It has been shown that alterations of the balance between activators and pro-MMP-9 can result in pro-MMP-9 activation under controlled conditions in cultured cells [51–53]. This is essential to produce catalytically competent activators and to overcome the effects of protease inhibitors [29,51]. Finally, the interaction between pro-MMP-9 and its activator should be optimal for the activation process to take place. We postulate that due to the free diffusion of pro-MMP-9 in the culture supernatant, pro-MMP-9 association with potential activators is severely restricted. Although various mechanisms for the association of pro-MMP-9 with the cell surface have been described, including CD44 [3] and the α2(IV) chain [21], none have so far been associated with zymogen activation [54]. Furthermore, in cultured cells, any possible activation of surface bound pro-MMP-9, if existent, would be further hampered by the rapid dissociation of the zymogen from the cell surface into the supernatant. Indeed, the bulk of pro-MMP-9 in cultured cells is present in the supernatant. Because of this reason, our attempts to follow surface activation of pro-MMP-9 by various activators including MMP-2 and MMP-3 in cultured cells yielded negative results (unpublished observations from our laboratory). Here, using isolated PM fractions containing MT1-MMP and TIMP-2 and ultracentrifugation, we have shown that presence of the PM milieu promoted the activation of pro-MMP-9 by MMP-2 when compared to the partial activation observed with MMP-2 released into the soluble fraction. Also, the activation of pro-MMP-9 by MMP-3 was enhanced by the presence of PM from MCF10A cells. Considering that MMP-3 is an efficient pro-MMP-9 activator in solution, the effect of the PM is significant and suggests that the PM scaffold may represent a favorable microenvironment for the interaction of pro-MMP-9 with potential activators that are not reproduced under the free diffusion conditions of culture supernatant. Since MMP-2 is less efficient than MMP-3 in promoting pro-MMP-9 activation, the effect of the PM microenvironment may be more relevant. We postulate that concentration of the activation complex (MT1-MMP/MMP-2) on the tumor pericellular space would favor the activation of pro-MMP-9 by MMP-2. Because both the gelatinases and MT1-MMP are usually co-expressed in many tumors, this mechanism of pro-MMP-9 activation, albeit not exclusive, is likely to be physiologically relevant and a major mediator of proteolysis at the pericellular space.

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Appendix 7

2

Surface Association of Secreted Matrix Metalloproteinases

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I. Introduction

Proteolysis of the pericellular milieu is a fundamental physiological process by which cells modify their immediate microenvironment to achieve homeostasis and fulfill their biological destiny. The pericellular environment is a complex ensemble of surface components and extracellular matrix (ECM) proteins and any change in its composition and structure has a profound impact on cell behavior and survival. The task of adjusting the nature and composition of the pericellular milieu in the organism relies on specific proteases produced by a variety of cells in the tissue. The tight control of proteolytic activity produces optimal cellular responses while uncontrolled activity causes significant tissue damage. Various proteolytic systems are at the cell's command to carry out surface proteolysis.

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One of the major groups of proteases responsible for pericellular proteolysis is the matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases. The MMP family comprises at least 27 members in vertebrates (Massova *et al.,* 1998; Nagase and Woessner 1999; Sternlicht and Werb, 2001), which are divided into five major subgroups based on their substrate specificity and structural organization: collagenases, stromelysins, matrilysins, gelatinases, and membrane type-MMPs (Massova *et al.,* 1998). MMP-19 and MMP-23 do not fall into these categories and thus may represent new subfamilies.

The members of the MMP family are key mediators of pericellular proteolysis in many physiological and pathological conditions, and in the past 20 years a considerable effort has been put forth to understand their mechanism of action and inhibition. Targeting the MMPs may interfere with the pathogenesis of various human diseases characterized by uncontrolled degradation of the ECM such as cardiovascular diseases, arthritis, and cancer. Therefore, a vast number of synthetic MMP inhibitors were developed and tested in animal models and in human clinical trials for effectiveness. Although some MMP inhibitors have shown promise, the targeting of MMPs is hampered by the structural similarities among the members of the MMP family, which reduces selectivity. The MMPs possess considerable structural similarities in their individual domain structures, in particular in the catalytic and hemopexin-like domain (Massova *et al.,* 1998), and with few exceptions, they have a considerable overlap in substrate specificity (Sternlicht and Werb, 2001). Biologically, the members of the MMP family also exhibit a high degree of redundancy in their cellular function. For example, studies investigating the role of MMPs in tumor cell migration and invasion have shown that these processes can be equally mediated by a variety of both secreted and membrane-anchored MMPs. Furthermore, knockout mice deficient in MMP genes, with the exception of MT1-MMP (Holmbeck *et al.,* 1999; Zhou *et al.,* 2000), showed no significant phenotypes when unchallenged (Parks and Shapiro, 2001). Also, the evidence shows that in most physiological and pathological conditions there is a consistent coexpression of various MMPs with overlapping properties. From the drug design perspective, these properties of the MMPs represent a major challenge. From the biological standpoint, the similarities between MMPs raise a fundamental dilemma on regulation of pericellular proteolysis: how do cells execute their proteolytic programs given the vast number of MMPs at the cell's command? Obviously, differential tissue expression and presence of tissue inhibitors of metalloproteinases (TIMPs), a family of natural MMP inhibitors, afford the cells the ability to control MMP-dependent proteolysis in tissues. However, these regulatory mechanisms, albeit important, do not explain how do cells can control the activity of MMPs at the pericellular space. New evidence indicates that focusing the activity of MMPs at the cell surface represents an additional level of regulation that permits cells to strictly control the initiation, inhibition, and termination of proteolytic activity on the cell surface. Furthermore, surface proteolysis affords the cells the ability to hydrolyze biologically relevant molecules in their immediate milieu.

To concentrate MMP activity at the cell surface, the MMP family developed unique structural (domain additions) and non structural features (recruitment of binding proteins) and hence today the MMPs can be subdivided in two major groups: the secreted and the membrane-anchored MMPs. A subgroup of MMPs incorporated membrane-anchoring motifs and thus evolved as membrane type-MMPs (MT-MMPs), which are the topic of Chapter 1. On the other hand, the secreted MMPs recruited various cell membrane proteins to act as docking sites on the cell surface. Many studies reported the association of secreted MMPs with the cell surface in various cell types including primary, nonmalignant, and malignant cells (Table I). The presence of secreted MMPs on the cell surface was investigated using a variety of techniques including isolation of purified plasma membranes and flow cytometry. These findings led to the search for specific surface components responsible for the MMP binding, which culminated with the identification of

Table I Reported Cell Surface Association of Secreted MMPs

Enzyme	Cell Type	Reference
MMP-9	Human pancreatic cancer RWP-I cells	Zucker *et al.*, 1990
	Human breast epithelial MCF10A cells	Toth *et al.*, 1997
	Bovine microvascular endothelial cells	Partridge *et al.*, 1997
	Human fibrosarcoma HT1080 cells[a]	Ginestra *et al.*, 1997
	Human neutrophils	Gaudin *et al.*, 1997
	Human breast carcinoma 8701-BC cells[a]	Dolo *et al.*, 1998
	Rat vascular endothelial cells	Olson *et al.*, 1998
	Murine mammary carcinoma Met-1 cells	Bourguignon *et al.*, 1998; Yu and Stamenkovic, 1999
	Human ovarian carcinoma CABA I cells[a]	Dolo *et al.*, 1999
	Prostate cancer DU-145 cells	Manes *et al.*, 1999
	Breast cancer MCF7 cells	Mira *et al.*, 1999
	Murine mammary carcinoma TA3 cells	Yu and Stamenkovic, 1999
	Prostate cancer PC3 cells	Festuccia *et al.*, 2000
	Normal mouse keratinocytes	Yu and Stamenkovic, 2000
	Human ovarian carcinoma OVCA 429 cells	Ellerbroek *et al.*, 2001
MMP-13	Rat osteosarcoma UMR 106-1	Barmina *et al.*, 1999
	Rat osteosarcoma ROS 17/2.8	Barmina *et al.*, 1999
	Normal rat osteoblasts	Barmina *et al.*, 1999
	Normal rat embryo fibroblasts	Barmina *et al.*, 1999
MMP-1	Rat mammary carcinoma BC1-5 cells	Whitelock *et al.*, 1991
	Human lung carcinoma LX-1	Guo *et al.*, 2000
	Primary human keratynocyttes	Dumin *et al.*, 2001
MMP-7	Human neuroblastoma SH-SY5Y	Yu *et al.*, 2002
MMP-19	Human peripheral blood mononuclear cells	Sedlacek *et al.*, 1998
	Human myeloid THP-1 cells	Mauch *et al.*, 2002
	Human myeloid HL-60 cells	Mauch *et al.*, 2002

[a]Presence in shed vesicles.

Table II Identified Proteins Associated with Binding of Secreted MMPs

Enzyme	Binding Protein	Function
MMP-9	$\alpha 2$ chain of collagen IV (Olson *et al.*, 1998)	Surface association and matrix localization
	CD44 hyaluronan receptor (Bourguignon *et al.*, 1998; Yu and Stamenkovic, 1999)	Surface association
	RECK (Takahashi *et al.*, 1998)	Inhibition of expression and activity
	LRP (Hahn-Dantona *et al.*, 2001)	Internalization of zymogen
MMP-1	$\alpha_2\beta_1$ integrin (Dumin *et al.*, 2001)	Localization at cell-collagen binding sites
	EMMPRIN (Guo *et al.*, 2000)	Surface association
MMP-13	Endo180? (Barmina *et al.*, 1999)	Internalization
MMP-7	Heparan sulfate proteoglycan (Yu and Woessner, 2000)	Matrix localization
	CD44/HSPG (Yu *et al.*, 2002)	Surface association

several MMP-binding proteins, each acting to control various aspects of enzyme function (Table II). Today, the surface association of MMPs is no longer viewed as a mere physical docking of the protease on the cell surface but a complex array of cell–protease interactions that regulate enzymatic activity and substrate preference. Indeed, new evidence shows that surface binding of MMPs provides the cells with the capability to process a vast spectrum of surface precursor molecules that, once activated, play pivotal roles in normal cellular and tissue functions (Sternlicht and Werb, 2001). On the other hand, surface binding also allows cells to terminate proteolysis by either inhibition (Takahashi *et al.*, 1998) or internalization of extracellular proteases (Hahn-Dantona *et al.*, 2001; Walling *et al.*, 1998). As later discussed in this review, these mechanisms allow the cells to fine-tune the control of closely homologous proteases such as the MMPs by adding an extra level of regulation involving subcellular localization. This review summarizes our current knowledge of the surface association of those secreted MMPs on which there is information available. MMP-2 (gelatinase A) is not included in this chapter, and its surface binding is discussed in Chapter 1.

II. Surface Binding of MMP-9

A. Overview

MMP-9 (gelatinase B), together with MMP-2 (gelatinase A), belongs to the gelatinase subfamily of MMPs, which comprise a distinct subgroup of MMPs characterized by unique structural features, interactions with TIMPs, and substrate specificity (Collier *et al.*, 1988; Wilhelm *et al.*, 1989). At the domain organization

level, MMP-9, in addition to the basic domains of all MMPs, contains a so-called gelatin-binding domain (GBD) inserted within the catalytic domain, which consists of three tandem copies of 58 amino acid residues, each homologous to the fibronectin type II-like module (Murphy and Crabbe, 1995). The GBD mediates the binding of the latent and active gelatinases to denatured collagen, also known as gelatin (Strongin *et al.,* 1993). Consistently, the substrate profile of the gelatinases demonstrates that these enzymes are efficient gelatin-degrading proteases and thus are thought to accomplish the later steps of collagen degradation by carrying out hydrolysis of collagen molecules after the attack of interstitial collagenases. However, MMP-2 was also shown to exhibit classical collagenase activity (Aimes and Quigley, 1995). The gelatinolytic activity of the gelatinases has been exploited as a means to identify these enzymes in biological samples including cell surfaces using the gelatin zymography technique (for a detailed review of zymography, see Toth and Fridman, 2001). Several unique features confer MMP-9 with distinct characteristics. For example, MMP-9 contains an additional 54-amino-acid long, proline-rich extension with homology to the α2(V) chain of collagen V that is located between the hinge region and the hemopexin-like domain (Wilhelm *et al.,* 1989). Furthermore, MMP-9, as opposed to MMP-2, is heavily glycosylated with both N- and O-linked glycosylation sites, which comprise a significant portion of the total molecular mass (Opdenakker *et al.,* 2001a). The function of the oligosaccharide moieties of MMP-9 remains unknown. In biological samples, MMP-9 is found in three distinct forms: a monomer, a disulfide-linked homodimer (Olson *et al.,* 2000; Strongin *et al.,* 1993), and as a covalent complex with lipocalin (Kjeldsen *et al.,* 1993; Yan *et al.,* 2001). Although both the monomeric and dimeric forms of MMP-9 are fully competent proteases, the zymogenic form of the dimer exhibits a significantly slower rate of activation by MMP-3 when compared to the monomeric form (Olson *et al.,* 2000). The MMP-9–lipocalin complex has been shown to play a role in potentiation of zymogen activation (Tschesche *et al.,* 2001) and to protect MMP-9 from degradation (Yan *et al.,* 2001).

Like all MMPs, active MMP-9 is inhibited by the TIMPs, which bind to the catalytic site with high affinity resulting in complete inhibition of enzymatic activity (Olson *et al.,* 1997). The latent form (referred to here as pro-MMP or progelatinase) of MMP-9 can form noncovalent complexes with TIMP-1, which are mediated by specific molecular interactions between the hemopexin-like domain of the enzyme and the C-terminal region of the TIMP (Strongin *et al.,* 1993). Pro-MMP-9 can also bind TIMP-3 (Butler *et al.,* 1999). The ability to generate zymogen/inhibitor complexes is a unique feature of the gelatinases among the members of the MMP family. The zymogen/inhibitor complex of pro-MMP-2 with TIMP-2 is involved in surface association and activation (Butler *et al.,* 1998; Strongin *et al.,* 1995), whereas the role of the pro-MMP-9/TIMPcomplexes remains undefined.

MMP-9 has been extensively studied in the context of its role in tumor metastasis and angiogenesis (Coussens *et al.,* 2000). MMP-9 has also been shown to play a critical role in the degradation of ECM in cardiovascular diseases (Ducharme *et al.,* 2000). Mice deficient in MMP-9 display an abnormal pattern of skeletal

growth plate vascularization and ossification (Vu *et al.,* 1998). MMP-9 is expressed by various types of cells including endothelial, epithelial, fibroblast, and immune cells, but the major producers of MMP-9 in tissues are the inflammatory cells (Opdenakker *et al.,* 2001b).

Most of the studies describing the association of MMP-9 with the cell surface were carried out with cell lines exposed to physiological and nonphysiological MMP-9 inducers such as growth factors (Ellerbroek *et al.,* 2001; Manes *et al.,* 1999; Mira *et al.,* 1999) and phorbol ester (Ginestra *et al.,* 1997; Mazzieri *et al.,* 1997; Olson *et al.,* 1998; Toth *et al.,* 1997). Upon secretion, MMP-9 is usually released into the culture media in a latent form. However, a fraction of the enzyme pool can be detected on the cell surface (Ellerbroek *et al.,* 2001; Festuccia *et al.,* 2000; Manes *et al.,* 1999; Mira *et al.,* 1999; Toth *et al.,* 1997) and in purified plasma membrane fractions (Toth *et al.,* 1997; Zucker *et al.,* 1990). Surface-bound MMP-9 can be readily extracted with aqueous solutions consistent with the fact that MMP-9 is a peripheral plasma membrane-associated protein (Toth *et al.,* 1997). MMP-9 was identified on the surface of a variety of cultured cells including endothelial (Olson *et al.,* 1998; Partridge *et al.,* 1997), breast epithelial (Olson *et al.,* 1998; Toth *et al.,* 1997), breast cancer (Mira *et al.,* 1999), pancreatic cancer (Zucker *et al.,* 1990), ovarian cancer (Ellerbroek *et al.,* 2001), prostate cancer (Festuccia *et al.,* 2000), fibrosarcoma (Mazzieri *et al.,* 1997), and mouse mammary carcinoma (Yu *et al.,* 1999) cells. Other studies showed the presence of MMP-9 in shed plasma membrane vesicles of human fibrosarcoma (Ginestra *et al.,* 1997) and endothelial (Taraboletti *et al.,* 2002) cells. These studies helped to establish the concept that MMP-9 is also a surface-associated protease and led to the search for MMP-9 binding proteins.

B. Binding of MMP-9 to the α2(IV) Chain

The α2(IV) chain of collagen IV was the first protein to be identified as an MMP-9 binding surface protein (Olson *et al.,* 1998). Ligand binding studies of iodinated pro-MMP-9 in breast epithelial MCF10A cells showed that MMP-9 binds to the cell surface with high affinity ($K_d \sim 22$ nM) (Olson *et al.,* 1998), which suggested the presence of a specific MMP-9 binding molecule. To identify the putative MMP-9 binding molecule, cell extracts of surface biotinylated-MCF10A breast epithelial cells were subjected to an MMP-9-affinity purification and a major biotinylated protein of 190 kDa was identified (Olson *et al.,* 1998). Sequencing data and specific antibodies demonstrated that the 190-kDa protein was the α2(IV) chain of basement membrane collagen IV. Coimmunoprecipitation experiments further confirmed the α2(IV) chain as the major MMP-9 binding protein in various cell types including breast epithelial MCF10A, breast carcinoma MDA-MB-231, fibrosarcoma HT1080, and rat vascular endothelial cells (Olson *et al.,* 1998; Toth *et al.,* 1999). These finding were consistent with immunohistochemical studies

showing the presence of MMP-9 on the surface of cancer cells in breast carcinomas (Visscher *et al.,* 1994) and in the basement membrane of skin tumors (Coussens *et al.,* 1999; Karelina *et al.,* 1993). Formation of the pro-MMP-9/TIMP-1 complex did not preclude binding of pro-MMP-9 to the α2(IV) chain, suggesting that the site of interaction is not the hemopexin-like domain, which is responsible for binding TIMP-1 (Olson *et al.,* 1998). The gelatinases bind to denatured collagens via the gelatin-binding domain, which exhibits a high degree of homology between the two enzymes (Murphy and Crabbe, 1995). However, pro-MMP-2 showed a significantly lower affinity for the α2(IV) chain when compared to pro-MMP-9 (Olson *et al.,* 1998). Possibly, other sites and/or the enzyme conformation regulate the interactions with the α2(IV) chain.

Functionally, the binding of MMP-9 to the α2(IV) chain does not appear to play a direct role in the regulation of zymogen activation and/or enzyme inhibition (Olson *et al.,* 1998). It is also unknown whether the activity of MMP-9 is altered in any way by this interaction. However, the fact that MMP-9 is an efficient gelatinolytic enzyme suggests that the binding of MMP-9 to the α2(IV) chain plays a role in the degradation of the collagen IV network at the cell surface. If so, the discovery of the α2(IV) chain is not surprising given the well-known ability of the gelatinases to bind with high affinity to denatured collagens by means of the gelatin binding domain (Allan *et al.,* 1995). Consistently, the gelatinases are very efficient gelatinolytic enzymes (Murphy and Crabbe, 1995), which are considered to participate in the degradation of the collagen matrix subsequent to the action of collagenolytic enzymes such as MMP-1, MMP-13, or MT1-MMP. Consistent with this view, the gelatinases exhibit a weak affinity for native (trimeric) collagens (Olson *et al.,* 1998; Steffensen *et al.,* 1998). It is conceivable that the binding of MMP-9 to the α2(IV) chain is mediated by sites that are cryptic in native collagen IV, which are only exposed after partial denaturation and/or degradation of the collagen IV molecule. If so, the binding of pro-MMP-9 to the α2(IV) chain uncovers a potential proteolytic mechanism that regulates the remodeling of basement membrane collagen IV by localizing the zymogen at a precise location where upon activation completes the breakdown of the collagen network.

Although both gelatinases can cleave denatured collagen IV with similar efficiencies, MMP-9 exhibits a higher affinity toward the α2(IV) chain (Olson *et al.,* 1998), suggesting that the association of MMP-9 with α2(IV) may serve a different purpose. Thus, beyond the potential role of the pro-MMP-9/α2(IV) complex in degradation of denatured collagen IV, the existence of single α2(IV) chains in the extracellular space raises the interesting possibility that α2(IV) chains may serve as pro-MMP-9 binding proteins on the cell surface. However, the stability of monomeric α2(IV) chains remains controversial (Toth *et al.,* 1999; Yoshikawa *et al.,* 2001). This fact does not minimize the potential significance of the high-affinity binding of MMP-9 to α2(IV) and consequently its association with collagen IV. Because collagen IV molecules are very closely associated with the cell plasma membrane, most likely bound via integrins, a clear distinction between cell

surface and ECM cannot be made. It is therefore permissible to consider the site of MMP-9 interaction with collagen IV as pericellular rather than extracellular. This pericellular milieu constitutes the biological front where relevant proteolysis must take place.

C. Binding of MMP-9 to CD44

A series of studies (Bourguignon *et al.,* 1998; Yu and Stamenkovic, 1999) showed the association of MMP-9 with the hyaluronan receptor CD44. The adhesion receptor CD44 is a heavily glycosylated transmembrane protein that as a consequence of extensive alternative splicing exists in multiple variant forms. Numerous studies have implicated CD44 in tumor growth, invasion, and metastasis (Goodison *et al.,* 1999) and thus, the association of MMP-9 with CD44 has been suggested to link cellular adhesion to ECM and pericellular proteolysis (Yu and Stamenkovic, 1999). Studies with murine mammary carcinoma and human melanoma cells expressing CD44 reported the presence of MMP-9 on the cell surface (Bourguignon *et al.,* 1998; Yu and Stamenkovic, 1999). The association of MMP-9 with CD44 was demonstrated by coimmunoprecipitation experiments and colocalization of proteins on the cell surface by immunofluorescence (Bourguignon *et al.,* 1998; Yu and Stamenkovic, 1999), but the relative binding affinity of MMP-9 toward CD44 remains unknown. Analyses of the surface-bound MMP-9 by gelatin zymography revealed presence of the active form as determined by molecular mass (Yu and Stamenkovic, 1999), suggesting that CD44 interacts specifically with the active species of MMP-9. However, it is unclear whether binding to CD44 plays a role in pro-MMP-9 activation. The binding of MMP-9 to CD44 appears to be associated with the ability of CD44 receptors to aggregate on the cell surface and binding of CD44 to hyaluronan is not necessary for the association of MMP-9 to CD44. However, hyaluronan induces coclustering of CD44 receptors with active MMP-9 in the mouse mammary cell line.

Functionally, the association of MMP-9 with CD44 has been shown to promote tumor cell invasion *in vitro* and in experimental metastasis assays (Yu and Stamenkovic, 1999). Furthermore, expression of a soluble CD44 receptor abrogated invasion *in vitro* and *in vivo* and inhibition of MMP-9 activity by an inhibitory antibody or antisense technology obliterated the invasive ability of the cells expressing CD44 and surface-bound MMP-9 (Yu and Stamenkovic, 1999). Although surface-associated MMP-9 may focus ECM degradation on the immediate cellular environment, active MMP-9 bound to the CD-44 receptor was also shown to process latent tumor growth factor-β (TGF-β) to the biologically active form (Yu and Stamenkovic, 2000). Generation of active TGF-β on the cell surface may enhance tumor growth and metastasis by promoting a degradative phenotype (Overall *et al.,* 1989) and by inducing angiogenesis (Yu and Stamenkovic, 2000).

D. Binding of MMP-9 to RECK

A negative mechanism of MMP-9 expression and activity has been reported to involve the action of the membrane-anchored glycoprotein RECK (Takahashi *et al.,* 1998). The *RECK* gene was identified after screening genes that could suppress the transformed phenotype induced by the *ras* oncongene in mouse fibroblasts (Takahashi *et al.,* 1996). *RECK* codes for a glycosylphosphatidylinositol (GPI)-anchored cysteine-rich glycoprotein containing serine protease inhibitor-like domains and regions with weak homology to epidermal growth factor (EGF) (Takahashi *et al.,* 1998) and its expression is downregulated in a variety of tumor cell lines and in cells transformed by a variety of oncogenes. Expression of the recombinant RECK protein in various cell lines inhibited *in vitro* invasion and metastasis *in vivo* without significant effects on the proliferative capacity of the cells. Subsequently, it was found that the expression of RECK was associated with specific reduction in the amounts of secreted pro-MMP-9 while no differences were observed in pro-MMP-2 secretion. Although RECK has no homology to TIMPs, a soluble RECK protein was shown to inhibit the enzymatic activity of MMP-9 (Takahashi *et al.,* 1998), MMP-2, and MT1-MMP (Oh *et al.,* 2001), albeit with a lower affinity than TIMPs. The mechanism by which the RECK protein inhibits MMP activity is unclear given the homology of RECK to serine protease inhibitors, but the inhibitory effects are not specific since various MMPs, both soluble and membrane-anchored, were equally inhibited (Oh *et al.,* 2001). In contrast, there appears to be specificity in the recognition of pro-MMP-9 by a soluble recombinant RECK (Takahashi *et al.,* 1998). However, whether membrane-anchored RECK can bind pro-MMP-9 on the cell surface and regulate its activation remains unclear. The inhibitory effect of RECK is believed to be the cause of the tumor-suppressing effects of RECK and also for the lethal consequence of *RECK* gene ablation in knockout mice (Oh *et al.,* 2001). The *RECK* null mice fail to develop beyond the embryonic stage and exhibit severe malformation of mesenchymal and vascular tissues, which were ascribed to reduced MMP activity when compared to the wild-type *RECK* embryos. Proteins such as RECK have been proposed to represent a new class of protease inhibitors that act specifically on the cell surface to control enzymatic activity in the pericellular space, as opposed to soluble MMP inhibitors, which are secreted (Oh *et al.,* 2001). Thus, surface-bound molecules that can act as protease inhibitors may add an extra level of regulation during pericellular proteolysis by trapping and inhibiting secreted MMPs on the cell surface.

E. Binding of MMP-9 to LRP

The control of pericellular proteolysis is also mediated by an active process of internalization that eliminates proteolytic enzymes and inhibitors from the cell

surface. Low-density lipoprotein receptor-related protein (LRP) is a member of the LDL receptor family known to mediate the endocytotic intake of ligands as diverse as lipoproteins, protease–inhibitor complexes, proteases, growth factors, ECM components, viruses, and bacterial toxins (Herz and Strickland, 2001). More than 30 ligands were found to bind LRP with high affinity indicating the broad role that LRP plays in the regulation of protein function by internalization. LRP-mediated internalization of protease–inhibitor complexes has been shown to regulate the activity of urokinase-type plasminogen activator (uPA) and its receptor (uPAr) after inhibition by the plasminogen activator inhibitor (PAI)-1 (Nykjaer *et al.*, 1997). Internalization of the uPA/uPAr/PAI-1 complex permits recycling of uPAr molecules to the cell surface to maintain plasmin-dependent activity (Nykjaer *et al.*, 1997). Evidence suggests that LRP also plays a role in the control of MMP activity at the cell surface by mediating the internalization of various members of the MMP family. MMP-9 was shown to bind with high affinity to purified LRP either as a free enzyme or in complex with TIMP-1 (Hahn-Dantona *et al.*, 2001). Binding of MMP-9 to LRP was inhibited by RAP (receptor associated protein), a protein that inhibits ligand binding to LRP (Herz and Strickland, 2001). Upon binding to embryonic fibroblasts expressing LRP, the MMP-9/TIMP-1 complex was shown to be internalized as a function of time, a process that was followed by degradation in a chloroquine-dependent mechanism (Hahn-Dantona *et al.*, 2001). Based on these studies a role for LRP has been proposed in which LRP controls the level of MMP-9 in the pericellular environment by promoting the clearance and subsequent catabolism of the latent enzyme and the complex of pro-MMP-9 with TIMP-1. Thus, LRP may act as a negative regulator of MMP-9 action. In this regard, it is interesting that LRP internalizes the complex of pro-MMP-9 with TIMP-1, which is catalytically inactive. There is not yet evidence that LRP can promote the internalization of the active enzyme or the active enzyme in complex with TIMPs.

III. Surface Binding of the Soluble Interstitial Collagenases (MMP-1 and MMP-13)

A. Overview

The degradation of the connective tissue matrix is mediated by a limited number of specialized MMPs, which acquired the capacity of hydrolyzing native triple helical interstitial collagen molecules. These enzymes are efficient in promoting the degradation of fibrilar collagen molecules at a single locus in both the $\alpha 1$ and $\alpha 2$ chains and thus are known to generate the classical 3/4 and 1/4 degradation fragments. Cleavage of collagen I molecules causes instability of the triple helical structure and the denatured collagen becomes sensitive to the proteolytic attack

of gelatinases. Fibrilar collagen degradation is a fundamental process during the development and formation of connective tissues such as bone and cartilage and for the remodeling and turnover of the collagenous matrix in normal and pathological conditions that necessitate collagen degradation. Both membrane-anchored and soluble MMPs can cleave interstitial native collagen (I to III) molecules including MMP-1 (fibroblast collagenase, collagenase-1), MMP-13 (collagenase-3), MMP-14 (MT1-MMP), MMP-8 (neutrophil collagenase, collagenase-2), and MMP-2 (gelatinase A), albeit with various degrees of efficiency (Krane, 2001).

Degradation of interstitial collagens must be strictly regulated to prevent unnecessary tissue damage. Localization of collagenolytic activity at the cell–matrix interface would permit turnover and remodeling of the collagen matrix where it is really needed. It is evident that by being anchored to the plasma membrane MT1-MMP, a potent collagenolytic enzyme, can focus its collagenase activity at the cell surface. However, how do the soluble interstitial collagenases achieve localized degradation of fibrilar collagens? Recent studies have attempted to answer this question in regard to the surface regulation of MMP-1 and MMP-13.

B. Surface Binding of MMP-1

1. Binding to $\alpha_2\beta_1$ Integrin

MMP-1 is produced by a variety of cell types and its major role is to promote the degradation of native collagen I molecules in the interstitial connective tissue matrix. MMP-1 is particularly active in normal and pathological processes that require turnover and remodeling of fibrilar collagen, particularly during development and wound healing, but it has also been associated with tumor invasion and metastasis (Brinckerhoff *et al.*, 2000). The activity of MMP-1 during wound healing must be efficiently and tightly regulated so that the proteolytic events produce the appropriate cellular responses and accomplish effective tissue repair. A series of studies in human keratinocytes, the major cell of the skin, elegantly demonstrated that the latent form of MMP-1 associates with the cell surface via the $\alpha_2\beta_1$ integrin (Dumin *et al.*, 2001; Stricker *et al.*, 2001). The $\alpha_2\beta_1$ integrin is the major collagen-binding receptor and is known to mediate the adhesion of keratinocytes to collagen I. Coimmunoprecipitation experiments, cellular colocalization, and solid-phase binding studies showed that pro-MMP-1 interacts with and binds specifically to $\alpha_2\beta_1$ integrin. Indeed, $\alpha_3\beta_1$, another integrin receptor for collagen I, was not detected in complex with MMP-1. MMP-1 binding to $\alpha_2\beta_1$ integrin was shown to be mediated by the I domain of the $\alpha 2$ integrin subunit and the hinge region and hemopexin-like domain of pro-MMP-1 (Dumin *et al.*, 2001; Stricker *et al.*, 2001). Solid-phase assay studies with purified α_2 I domain and MMP-1 (active and latent) showed a high-affinity (around 10–40 nM) binding for both enzyme forms, although the zymogen appeared to bind preferentially (Stricker *et al.*, 2001). Under

the experimental conditions used, the interaction of pro-MMP-1 with $\alpha_2\beta_1$ was evident when the cells were plated on native collagen I and was significantly reduced on gelatin. This observation suggested that the nature of the matrix influences the ability of the $\alpha_2\beta_1$ integrin to cluster pro-MMP-1 on the cell surface.

The significance of the binding of pro-MMP-1 to $\alpha_2\beta_1$ has been associated with the ability of keratinocytes to migrate on collagen I during the process of wound healing in the skin. In response to injury, keratinocytes on the epidermal layer migrate to the underlying collagen I-rich connective tissue matrix to initiate the process of tissue repair. Expression of $\alpha_2\beta_1$ at the basal surface of the migrating keratinocytes confers on the cells the ability to form focal contacts with the collagen matrix, which in turn transduce extracellular signals that stimulate the repair process by inducing the expression of proteolytic enzyme such as MMP-1, among other factors (Sudbeck *et al.,* 1997). Dumin *et al.* (2001) proposed that binding of MMP-1 to $\alpha_2\beta_1$ would promote the generation of denatured collagen I molecules at areas of focal contacts. This process would cause a reduction in the affinity of $\alpha_2\beta_1$ toward collagen resulting in the dislodging of the integrin from its binding to the now-denatured collagen. This would allow the cells to detach and generate new contacts at the leading front, in the uncleaved collagen I matrix. In addition, hydrolysis of collagen I at focal contacts by MMP-1 would elicit a negative feedback on enzyme gene expression and binding to $\alpha_2\beta_1$ (Dumin *et al.,* 2001). This hypothesis was based on the observation that induction of pro-MMP-1 gene expression and binding to $\alpha_2\beta_1$ were more efficient when the cells were in contact with native collagen I (Dumin *et al.,* 2001). Paradoxically, Dumin *et al.* (2001) found that the zymogen form of MMP-1 was the major enzyme species that was consistently bound to $\alpha_2\beta_1$ and that pro-MMP-1 was the major form detected in the supernatant of keratinocytes plated on collagen I. These observations raise the question as to whether the binding of pro-MMP-1 to $\alpha_2\beta_1$ can regulate zymogen activation and whether the enzyme remains associated with the integrin after activation. Considering that the zymogen form is likely to be catalytically inactive, the detection of surface bound pro-MMP-1 (Dumin *et al.,* 2001) or pro-MMP-9 as described earlier (Ellerbroek *et al.,* 2001; Toth *et al.,* 1997) represents a major dilemma in our understanding of the relevance of cell surface association of the zymogen for cellular function. Recent evidence suggests that pro-MMP-9 exhibits limited enzymatic activity without removal of the propeptide if the enzyme is bound to gelatin (Bannikov *et al.,* 2002). Whether a similar process exists in pro-MMP-1 upon binding to $\alpha_2\beta_1$ and/or collagen I is still unknown. Further studies are required to support a functional role for the binding of pro-MMP-1 to $\alpha_2\beta_1$ on cell behavior or on enzymatic activity.

2. Binding to EMMPRIN/CD147

Invasive tumor cells migrate through the interstitial collagen matrix of the stroma to reach the circulation and thus they must possess the necessary proteolytic

machinery to degrade various collagen types during cell migration. There is ample evidence indicating that MMP-1 expression is elevated in various invasive tumors (Brinckerhoff *et al.,* 2000). Similar to the findings with human keratinocytes and MMP-1 (Dumin *et al.,* 2001), the concept of surface localization as a means to improve the effectiveness of collagen degradation can also be applied for MMP-1 and tumor cells. Supporting this view, early studies showed the presence of MMP-1 on the plasma membranes of tumor cells (Moll *et al.,* 1990). In recent years, however, production of proteolytic enzymes, including MMPs, has been shown to take place mostly in the stromal cells that are closely associated with the cancer cells (Crawford and Matrisian, 1994; Hewitt and Dano, 1996). The expression of MMPs by the stromal cells is a consequence of specific tumor–stromal interactions mediated by a variety of factors. One of this factors is the transmembrane protein EMMPRIN (extracellular matrix metalloproteinase inducer) or CD147/basigin (Biswas *et al.,* 1995), a member of the immunoglobulin superfamily that is found on the surface of many tumor cells and is known to induce the expression of several MMPs in fibroblasts (Guo *et al.,* 1997). For instance, tumor-bound EMMPRIN has been shown to stimulate MMP-1 expression in fibroblasts and thus may contribute to the degradation of the interstitial collagen matrix in tumor tissues. However, this degradative process, if limited to the fibroblast pericellular space, may have little impact on the immediate environment of the tumor cells and their ability to migrate. The tumor cells, to take advantage of the MMPs produced by the stromal cells, must be able to retain the released enzymes on their surface. A study has provided a possible answer to this dilemma by showing that EMMPRIN itself can act as "receptor" for pro-MMP-1 on the surface of the tumor cells (Guo *et al.,* 2000). In that study, phage display screening was used to search for EMMPRIN binding proteins. This resulted in the isolation of clones containing DNA sequences of human MMP-1 suggesting that EMMPRIN may act as a surface binding protein for MMP-1. The ability of EMMPRIN to bind MMP-1 was verified using various approaches and the zymogen form of MMP-1 was found to bind preferentially to EMMPRIN (Guo *et al.,* 2000). Evidence suggests that EMMPRIN-mediated induction of MMP expression also involves both heterotypic and homotypic cell–cell interactions resulting in MMP expression in both fibroblasts and tumor cells (Sun and Hemler, 2001). Thus, EMMPRIN may not only act to induce expression of MMPs in the neighboring fibroblasts but also to serve as a docking protein for pro-MMP-1 on the surface of the invasive tumor cells in either a paracrine or an autocrine fashion. The functional consequences of binding the MMP-1 zymogen to EMMPRIN for both enzyme and cellular functions remain to be elucidated.

C. Surface Binding of MMP-13

MMP-13, or collagenase-3, was originally identified in breast carcinomas using a genetic approach and has been associated with the invasive and metastatic

properties of various human tumors (Pendas *et al.,* 2000). MMP-13 has also been shown to play a major role in bone formation (Jimenez *et al.,* 2001) and in the pathogenesis of osteoarthritis (Neuhold *et al.,* 2001).

A series of studies demonstrated the binding of MMP-13 to the cell surface suggesting the existence of a specific receptor (Omura *et al.,* 1994). Studies in rat osteoblastic cells showed that secreted or exogenously added MMP-13 was gradually removed from the extracellular space by an active process of internalization of the enzyme. Ligand binding studies further demonstrated the existence of single high-affinity (nanomolar range) receptor (Omura *et al.,* 1994; Walling *et al.,* 1998). However, it turned out that the initial binding and subsequent internalization of pro-MMP-13 was mediated by two distinct surface components. The protein responsible for pro-MMP-13 internalization was identified as LRP. This was supported by the observation that LRP null fibroblasts, albeit able to bind pro-MMP-13, were unable to internalize the enzyme. Furthermore, RAP inhibited MMP-13 internalization consistent with a role for LRP in this process (Barmina *et al.,* 1999). A pro-MMP-13-binding protein with a relative mass of 170 kDa was isolated and identified as the transmembrane glycoprotein Endo180 (Barmina *et al.,* 1999). However, this study did not show evidence for the interaction (Barmina *et al.,* 1999). Later studies using various approaches and similar cell lines failed to confirm the role of Endo180 in pro-MMP-13 surface binding (Bailey *et al.,* 2002) and thus the nature of the putative pro-MMP-13 receptor remains elusive. The possible role of Endo180 as mediator of pro-MMP-13 cell surface binding is nevertheless interesting. Endo180 is a member of the C-type lectins of endocytic receptors, which presently include the macrophage mannose receptor, the phospholipase A_2 receptor, and the DEC-205/MR6 receptor (Sheikh *et al.,* 2000). Structurally, these receptors are type I transmembrane glycoproteins containing within the extracellular portion a cysteine-rich domain at the amino-terminal region followed by a collagen binding domain (fibronectin type II) and 8 to 10 consecutive carbohydrate recognition domains (Sheikh *et al.,* 2000). This family of proteins serves as endocytic receptors for extracellular glycoproteins in a process mediated by clathrin-coated vesicles, which is followed by lysosomal degradation (Sheikh *et al.,* 2000). Endo180 was also found to bind the urokinase-type plasminogen activator receptor (uPAr) in a process dependent on the presence of pro-uPA, and thus Endo180 is also known as the urokinase-type plasminogen activator receptor-associated protein (uPARAP) (Behrendt *et al.,* 2000). In addition, Endo180/uPARAP was shown to bind collagen V via its collagen-binding domain (Behrendt *et al.,* 2000). Although the precise role of Endo180/uPARAP in uPAr function remains unknown, the biological properties of the C type lectin suggest that it may play a role in the internalization of the uPA/uPAr complex similar to the role of LRP in this process (Engelholm *et al.,* 2001). The multiple interactions mediated by Endo180/uPARAP raise the interesting possibility that this lectin may serve as a link between serine proteases, MMPs, and collagen degradation on the cell surface. Although the proposed interaction of Endo180/uPARAP

with pro-MMP-13 has been disproved (Bailey *et al.,* 2002), it is worth noting that pro-MMP-13 is heavily glycosylated and thus may have the potential to interact with lectin type proteins on the cell surface to mediate its internalization. Interestingly, pro-MMP-9, which is also heavily glycosylated, is also internalized by LRP (Hahn-Dantona *et al.,* 2001) suggesting the possibility that glycosylation of soluble MMPs may serve as a recognition signal for endocytic receptors to deplete the pericellular space of excess zymogens before they undergo activation. For pro-MMP-13, surface binding may also be associated with activation (Bailey *et al.,* 2002; Knauper *et al.,* 1997). However, whether this process necessitates the presence of a specific cell surface receptor to bring about the zymogen in close association with its activator remains unknown.

IV. Surface Binding of MMP-7

A. Overview

MMP-7 (matrilysin-1) (Wilson and Matrisian, 1996; Woessner, 1995), together with MMP-26 (matrilysin-2) (de Coignac *et al.,* 2000; Park *et al.,* 2000; Uria and Lopez-Otin, 2000), is the shortest member of the MMP family containing only a signal sequence, a propeptide, and a catalytic domain. Structural studies suggested that MMP-7's minimal domain composition represents a retrograde process in MMP evolution and thus MMP-7 may be a relatively new MMP in which deletion of the hemopexin-like domain occurred late in evolution (Massova *et al.,* 1998). MMP-7 exhibits broad substrate specificity, which includes ECM and non-ECM proteins (Wilson and Matrisian, 1996; Woessner, 1995). Accumulating evidence indicates that MMP-7 cleaves various proteins known to be associated with the cell surface including E-cadherin (Noe *et al.,* 2001), tumor necrosis factor-α precursor (Gearing *et al.,* 1994; Haro *et al.,* 2000), and Fas ligand (Powell *et al.,* 1999). MMP-7 is involved in a variety of physiological and pathological processes including uterus involution (Woessner, 1996), tumor progression (Fingleton *et al.,* 1999; Wilson and Matrisian, 1996), apoptosis (Powell *et al.,* 1999), and microbial defense (Wilson *et al.,* 1999). Mice deficient in MMP-7 lack efficient antimicrobial defenses in the lung (Wilson *et al.,* 1999) and do not support intestinal tumorigenesis (Wilson *et al.,* 1997).

B. Binding of MMP-7 to Heparan Sulfate

Several studies demonstrated the ability of MMP-7 to bind heparan sulfate (HS), a major glycosaminoglycan of the ECM (Yu and Woessner, 2000). HS moieties associate with a protein core forming heparan sulfate proteolglycans (HSPGs), which are heterogeneous and ubiquitous components of the ECM, in particular

basement membranes, but are also expressed on cell surfaces in transmembrane proteins. The HS side chains are linear polysaccharides composed of alternating dissacharide units of either uronic acid or L-iduronic acid and D-glucosamine and are negatively charged because of the high content of sulfate groups (Esko and Lindahl, 2001). HSPGs interact with a large number of proteins and thus play key roles in many biological processes as diverse as tissue development, growth factor signaling, angiogenesis, and tumor metastasis (Forsberg and Kjellen, 2001; Iozzo and San Antonio, 2001; Sanderson, 2001), to name just a few. Thus, the association of MMP-7 with HS may link proteolytic activity with HSPG-mediated processes.

The association of MMP-7 with sulfated proteoglycans was inferred from the fact that the enzyme was extracted from rat uterus with various sulfated compounds and/or after heparitinase treatment. Further studies determined that MMP-7 colocalizes with HS in rat uterus and that they are similarly regulated during the estrous cycle (Yu and Woessner, 2000). MMP-7 and HS were detected on the surface of the epithelial cells lining the uterus glands. Although the relative affinity of MMP-7 toward the cell surface was not directly determined, affinity measurements were carried out with immobilized heparin. These studies showed a high-affinity binding of both the latent and active forms of MMP-7, although the latent form exhibited a stronger affinity. Structural analyses of the catalytic domain of rat MMP-7 revealed the existence of a putative heparin-binding site represented by a contiguous line of positive residues, which may be responsible for the tight binding of MMP-7 to HSPG (Yu and Woessner, 2000).

An isoform of the multifunctional CD44 hyaluronan receptor containing the alternatively spliced exon v3 can incorporate HS side chains and thus is considered a nonclassical HSPG (Iozzo, 2001). Functionally, CD44/HS exhibits some of the functions of classical surface-associated HSPG including the ability to bind fibroblast growth factor (FGF) and heparin-binding epidermal growth factor (HB-EGF) (Bennett *et al.,* 1995). One study reported the association of CD44/HS with MMP-7 on the cell surface by colocalization, coimmunoprecipitation, and heparin elution studies (Yu *et al.,* 2002). The active form of MMP-7 appears to be preferentially associated with the heparan sulfate–containing CD44 isoform. The significance of this finding was then explained by the ability of MMP-7 to cleave the precursor form of heparin-binding epidermal growth factor (pro-HB-EGF) to generate active EGF. This growth factor can bind to CD44/HS and thus becomes accessible to the proteolytic processing of MMP-7, as demonstrated after coprecipitation of CD44/HS with pro-HB-EGF and exposure of the precipitated growth factor to MMP-7. Furthermore, it was shown that the mature HB-EGF could engage its receptor ErbB4 and directly affect organ remodeling in uterus and mammary glands (Yu *et al.,* 2002). These studies demonstrated that the association of active MMP-7 with the cell surface via CD44/HS can promote the hydrolysis of cell surface molecules and thus directly influence cell behavior. The findings with CD44/HS and MMP-7 raise interesting possibilities for other secreted MMPs, as

they have also been shown to bind to heparin (Butler *et al.,* 1998). Furthermore, TIMPs are also known to exhibit tight binding to heparin, which has been used for their isolation and purification. TIMP-3 has been shown to bind to HSPG in rat uterus in a similar fashion as MMP-7 (Yu *et al.,* 2000), suggesting the possibility that their close interaction may serve to regulate MMP-7 activity in the ECM and on the cell surface. The ability of HS chains to bind MMPs and TIMPs suggests the possibility that other HSPGs such as syndecans and glypicans could also act as potential cell surface binding proteins for these molecules and thus regulate pericellular proteolysis.

V. Surface Binding of MMP-19

MMP-19 is a secreted new member of the MMP family that was identified by searching databases containing expressed sequence tags (Cossins *et al.,* 1996; Pendas *et al.,* 1997) and by cDNA library screening (Kolb *et al.,* 1997) and was originally designated MMP-18 (Cossins *et al.,* 1996) and RASI-1 (Kolb *et al.,* 1997). Structurally, MMP-19 contains the basic domains of all MMPs including a signal peptide, a prodomain, a catalytic domain, and a hemopexin-like domain (Pendas *et al.,* 1997). However, the absence of several common structural features present in other MMP subfamilies makes MMP-19 a unique enzyme (Pendas *et al.,* 1997). MMP-19 contains two unique features including a hinge rich in acidic residues and a threonine-rich region of 36 residues at the end of the hemopexin-like domain downstream of the terminal cysteine (Cossins *et al.,* 1996; Pendas *et al.,* 1997). The catalytic competence of MMP-19 has been characterized with a recombinant catalytic domain, which was found to cleave a variety of ECM components, in particular collagen IV, from the basement membrane, laminin, and nidogen but shows no activity against triple helical collagens (Stracke *et al.,* 2000). MMP-19 is highly expressed in the synovial blood vessels of rheumatoid arthritis (Kolb *et al.,* 1997) and in myeloid cells (Mauch *et al.,* 2002). MMP-19 is expressed in normal breast tissues in myoepithelial, endothelial, and smooth muscle cells. Interestingly, compared to normal and noninvasive breast tumors, invasive breast carcinomas contain lower levels of MMP-19, suggesting that this enzyme may play a role in early stages of tumor progression.

Evidence indicates that MMP-19 associates with the cell surface of myeloid cells including activated blood mononuclear cells and various myeloid cell lines as determined by flow cytometry and surface biotinylation (Mauch *et al.,* 2002). The surface association of MMP-19 appears not to be mediated by a putative GPI anchor, and deletion of the hemopexin-like domain impaired surface association (Mauch *et al.,* 2002). The precise mechanism of MMP-19 surface association remains unclear but the presence of the C-terminal threonine-rich region may play a role in surface binding. Furthermore, the significance of the surface association remains to be determined.

VI. Concluding Remarks. Surface Binding: A Balance between Positive and Negative Effects on Pericellular Proteolysis

It is now evident that the surface association of secreted MMPs is a multifunctional process mediated by diverse surface proteins and involving, so far, only a limited number of secreted MMPs. It is also evident that binding of the secreted MMPs to the cell surface to be functionally meaningful for the cells does not require binding to a classical membrane-anchored molecule analogous to signaling or adhesion receptors. Instead, it may include surface molecules that facilitate clustering of the enzyme in the immediate pericellular microenvironment and surface proteins that can terminate proteolysis by removing the protease from the extracellular space. The differential effects of surface association on MMP function suggest that the fate of a surface-bound enzyme is determined by a balance between positive and negative elements. As shown in Fig. 1, binding of MMPs permits surface localization as demonstrated with the findings with MMP-9 (Olson *et al.*, 1998; Yu and Stamenkovic, 1999), MMP-1 (Dumin *et al.*, 2001), and MMP-7 (Yu *et al.*, 2002), and thus it may serve the cells to concentrate the secreted MMP at areas where

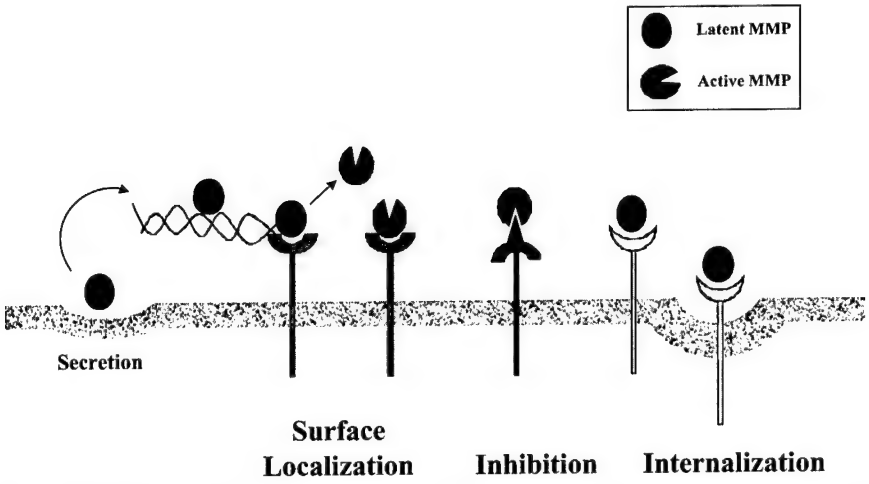

Figure 1 Distinct positive and negative aspects of surface association of secreted MMPs. The released MMPs can be retained on the cell surface by a variety of surface molecules including cell adhesion receptors or ECM components associated with the cell surface. This permits surface localization at areas of cell–matrix contact. Upon binding of the zymogen to these molecules, activation may ensue and the active enzyme may be released or remain bound. Alternatively, active enzyme may bind to a surface component. Binding to a surface-anchored protein with a protease inhibitory domain will result in inhibition of catalytic activity. Finally, surface binding may lead to enzyme internalization by endocytic receptors and thus terminate surface proteolysis. Interactions between these mechanisms and the action of TIMPs (not shown here) will determine the net proteolytic activity on the cell surface.

proteolysis is required. To achieve surface association, each of the secreted MMPs appears to utilize a different set of proteins suggesting a certain degree of specificity. If so, unique structural features in the secreted MMP molecules may target the enzyme to a specific surface-binding component. The presence of a gelatin-binding domain in the gelatinases is an example of adaptation for pericellular ECM binding. On the cells, however, the evidence so far indicates that there are not specific "receptors" for MMPs. Instead, surface proteins as different as cell adhesion receptors and ECM proteins have the ability to cluster secreted MMPs in areas of cell–matrix contacts. Although this association may facilitate substrate degradation at focal points, there is not yet evidence to indicate that such interactions regulate enzymatic activity. Further studies are required to determine the effects of surface binding on regulation of activation and catalytic activity of the secreted MMPs. If surface binding is required for efficient and localized control of enzymatic activity, a mechanism of inhibition that is also accessible to the pericellular environment must also exist to avoid excessive proteolysis. Membrane-anchored proteins with inhibitory domains such as RECK (Section II,E) (Takahashi *et al.,* 1998) may provide a tighter control of MMP activity on the cell surface (Fig. 1) by directly binding the active MMP or by inhibiting active enzyme bound to an MMP surface "receptor." On the other hand, surface binding of active enzyme may disrupt enzyme–TIMP binding and thus act as a sort of safe haven for catalysis at the cell's doorstep. Although this is an interesting concept, there is no experimental evidence to support such a possibility with surface-bound secreted MMPs. In fact, the MT-MMPs, which are tethered to the plasma membrane, are readily accessible and inhibited by TIMP-2, a secreted inhibitor. Thus, at this juncture, the effects of surface binding on secreted MMP-TIMP interactions cannot be predicted. Enzyme internalization is another component of the overall mechanism aimed at terminating enzymatic activity on the cell surface. This process can be mediated by proteins such as LRP as shown for MMP-9 (Hahn-Dantona *et al.,* 2001) and MMP-13 (Barmina *et al.,* 1999) but may include other surface molecules. The studies with MMP-13 suggest that cell surface binding and internalization are mediated by different components and thus a complex process may regulate binding and subsequent internalization. The balance between positive and negative elements determines the level of net enzymatic activity in the pericellular milieu and permits the cells to tailor the appropriate proteolytic response. Further studies into the mechanisms of surface regulation and the molecules involved in these processes may provide new avenues for the development of specific inhibitors of MMP action in pathological conditions.

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Appendix 8

Cell surface association of matrix metalloproteinase-9 (gelatinase B)

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Key words: matrix metalloproteinase, protease, cell surface binding, zymogen

Summary

Matrix metalloproteinase (MMP)-9 (gelatinase B) belongs to the MMP family of zinc-dependent endopeptidases that has been associated with tumor cell invasion and metastasis and tumor-induced angiogenesis. As a secreted MMP, pro-MMP-9 is released into the extracellular environment by both tumor and stroma cells, where it fulfills its proteolytic functions degrading both extracellular matrix (ECM) and non-ECM proteins. A major dilemma in our understanding of MMP-9 function is how the released protease is targeted to the right location and how its activity is controlled at the pericellular space. It has been proposed that MMP-9 interact with cell surface components and that this type of interaction positively regulates enzymatic activation and activity. However, recent evidence shows that association of MMP-9 with the cell surface is mediated by a distinct array of surface proteins that serve to regulate multiple aspects of the enzyme function including localization, inhibition and internalization. How these distinct mechanisms regulate the overall MMP-9 activity at the pericellular space remains an important goal in our understanding of MMP-9 function at the cell surface. Furthermore, the study of surface-associated MMP-9 imposes new conceptual and methodological challenges with particular consideration to the unique structural and functional characteristics of this key enzyme.

Introduction

Enhanced extracellular proteolytic activity is a characteristic of all malignant tumors that serves to promote tumor cell survival and dissemination. Tumor cells possess a vast arsenal of proteolytic enzymes at their disposal, which they utilize to control one or more aspects of the malignant process, including tumor growth, angiogenesis and metastasis. The MMPs constitute a family of zinc-dependent endopeptidases that are critical mediators of proteolytic events in tumor tissues. Among the members of the MMP family, pro-MMP-9, also known as gelatinase B, has been shown to be a key enzyme in tumor progression playing a pivotal role in tumor cell invasion and in angiogenesis [1–3]. Pro-MMP-9 expression is elevated in most human cancers and in tumor tissues; both tumor and stromal cells produce pro-MMP-9. As an ECM-degrading enzyme, pro-MMP-9 promotes degradation of a variety of ECM components and thus participates in the events that are required for tumor cell migration and tumor-induced angiogenesis. In recent years, the classic view of MMPs in tumor progression, in which the enzyme's major contribution to tumor invasion is to degrade the physical barriers that constitute the ECM, has been challenged [4]. Evidence has shown that several MMPs are involved in turnover and processing of proteins such as cell adhesion receptors, chemokines, growth factors, growth factor receptors and protease inhibitors [5,6]. In

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the case of MMP-9, various studies have shown that MMP-9 can directly accomplish cleavage of galectin-3 [7], transforming growth factor-β (TGF-β) [8] and plasminogen [9], three proteins that regulate tumor cell invasion and angiogenesis. Recently, MMP-9 has been implicated in the release of kit ligand from stromal bone marrow cells [10], a process that contributes to the proliferation of endothelial and hematopoietic stem cells. MMP-9 also can cleave tissue factor pathway inhibitor and thus influence coagulation [11]. Degradation of ECM by MMP-9 has been suggested to trigger an angiogenic response by promoting the release of ECM-bound vascular endothelial growth factor (VEGF) [2].

The substrates degraded by MMP-9 are in most cases present in the pericellular space, and therefore, there must be mechanisms to permit MMP-9 to achieve its full proteolytic potential at the cell surface. Thus, to be of use to the tumor, and regardless whether pro-MMP-9 is produced by tumor or stromal cells, extracellular proteolysis must take place in close association to the tumor cell surface [12]. Much effort has been put forward to understand how MMP-9 works at the cell surface and several mechanisms have been described. However, as will be discussed below, the binding of MMP-9 to the cell surface is not always associated with a positive effect on proteolysis. The evidence suggests that for MMP-9, surface association has not only evolved to bring the enzyme in close association with a particular pericellular substrate, but also to regulate the MMP-9 function at various levels including internalization and inhibition. This review will summarize the evidence linking MMP-9 to the cell surface and attempts to discuss some key issues when assessing MMP-9 surface association. A brief overview of the MMP-9 structure and activation will also be provided, as this information is relevant to understanding how MMP-9 may function at the cell surface.

Structural features of pro-MMP-9

The MMPs are multidomain zinc-dependent endopeptidases that are generally organized into four basic domains. These domains include a propeptide domain responsible for maintaining latency, a catalytic domain, a hinge (linker) region, and a *C*-terminal domain, also known as the hemopexin-like domain, which, depending on the given MMP, is necessary for substrate recognition and/or for binding to their physiological endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). The X-ray crystallographic structure of pro-MMP-9 (RCSB accession number 1L6J) was recently determined by Elkins et al. [13] (Figure 1(A)). The structure reveals the presence of three distinct domains: a propeptide, a gelatin-binding domain composed of a cluster of three fibronectin type II-like repeats and the catalytic domain (Figure 1(A)); not depicted in the structure are the signal peptide, the hinge domain and the hemopexin-like domain. The propeptide is positioned such that it faces the catalytic domain and blocks access to the active site. At its *N*-terminus, a loop protrudes towards a fibronectin type II-module and Phe^{31} (amino acid notation: pro-MMP-9_{1-707}) on that loop occupies a shallow hydrophobic pocket that is formed by residues Phe^{353}, Trp^{372}, Phe^{379}, Trp^{385} and Phe^{387} on the fibronectin type II-like module [13]. A cysteine residue at position 99 (within the propeptide) is found to coordinate with the active-site zinc ion. Upon activation of pro-MMP-9, it is known that this residue loses its coordination to the zinc ion, an event that ultimately leads to the release of the propeptide from the protein, resulting in a fully active enzyme (this is also known as the 'cysteine switch' mechanism [14,15]). Recent molecular dynamics and *ab initio* molecular orbital calculations [16] have shed light on the activation mechanisms of pro-MMP-2 and pro-MMP-9. It was found that after cleavage of the peptide bonds for the release of the propeptide, a water molecule would move into the active site and coordinate with the catalytic zinc ion, replacing the active-site glutamate carboxylate oxygen as the chelating agent (Glu coordinates the catalytic zinc ion after the cysteine switch has occurred) [16]. Additionally, *ab initio* molecular orbital calculations revealed that the cysteine should be protonated for the activation process to occur [16].

The X-ray structure of the catalytic domain of MMP-9 was first solved in a complex with an inhibitor by Rowsell et al. (RCSB accession number 1GKC) [17]. The recent structure of pro-MMP-9 by Elkins et al. [13] shows that the

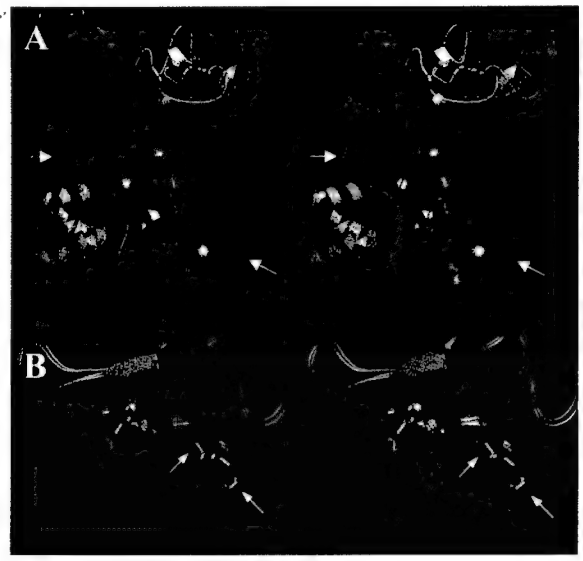

Figure 1. (A) Stereo view of the hemopexin-truncated pro-MMP-9 color-coded according to its various domains. The catalytic domain is shown in blue and the propeptide domain is depicted in green. The three fragments of the fibronectin type II-like domain are shown in white (domain 3), wheat (domain 1) and grey (domain 2). Metals are shown in sphere rendering with the zinc ions colored in orange and calcium ions colored in yellow. White and yellow arrows point to the *N*-glycosylation sites of MMP-9 (Asn^{38} and Asn^{120}, respectively). (B) A close-up stereo view of the superimposed active site domains of MMP-2 and MMP-9. The active site zinc (catalytic) is shown as a grey sphere. Also shown are the histidine residues that coordinate the catalytic zinc ion. All residues, including the active site histidines, are shown in capped sticks representation and color-coded according to atom types (white, blue and red for carbon, nitrogen and oxygen, respectively). White arrows point to residues Leu^{397} and Thr^{426} in MMP-9, respectively. The red arrow points to residue 424 in MMP-9, which is substituted by a threonine in MMP-2.

catalytic domain adopts a matrixin fold [18]. The secondary structure of the catalytic domain consists of five β-strands in a twisted β-sheet flanked by three α-helices (Figure 1A). The active site is sandwiched between the one β-strand (cyan in Figure 1B) and an α-helix (orange in Figure 1B) that holds two of the histidine residues (His^{401} and His^{405}) that are part of the conserved HExxHxxGxxH motif [18,19]. This α-helix is followed by a loop (colored in red in Figure 1B) that holds the third histidine (His^{411}) residue in the HExxHxxGxxH motif terminating into the methionine turn and leading into another loop (specificity loop) that runs along the large S1′ hydrophobic pocket [13]. The active-site histidines, His^{401}, His^{405} and His^{411} are tetrahedrally coordinated to the catalytic zinc ion. Another zinc ion is located in the active-site domain, and, like the calcium ions present in the catalytic domain, plays an important structural role (see Figure 1A for relative positions of zinc and calcium ions).

The structures of the fibronectin type II-like modules (gray, wheat and white in Figure 1A) consist of two short double-strand anti-parallel β-sheets, approximately perpendicular to each other and to three large irregular loops [13,14,20]. The structure of the *C*-terminal domain has been solved for MMP-1 [21], MMP-2 [14,22], MMP-13 [23] and recently for MMP-9 by Cha et al. [24]. The *C*-terminal hemopexin-like domain of MMP-9 consists of a four-bladed β-propeller structure with a pseudo-four-fold symmetry. The blades are composed of antiparallel four β-stranded β-sheet structures. Pro-MMP-9 can form a stable complex with either TIMP-1 [25] or TIMP-3 [26] via the *C*-terminal hemopexin-like domain, which in the case of TIMP-1 represents the high-affinity binding site [27]. Indeed, pro-MMP-9 is the only MMP that forms a high-affinity complex with TIMP-1. However, the role of the complex has not yet been elucidated.

The amino-acid sequence of pro-MMP-9 reveals that there are three potential *N*-glycosylation sites (Asn^{38}, Asn^{120}, Asn^{127}) and several *O*-glycosylation sites (the latter occurring in the hinge domain). A recent experimental and computational study by Kotra et al. [28] has shown that only two of the three potential *N*-glycosylation sites are indeed glycosylated, namely Asn^{38} and Asn^{120}, which are located in the propeptide and the catalytic domain respectively (see Figure 1A). Molecular dynamics simulations revealed that the oligosaccharides adopt stable conformations that do not lead to interactions with the protein beyond the point of attachment [28]. The hinge region of pro-MMP-9, located between the catalytic and the hemopexin-like domains consists of a proline-rich 56-amino acid long stretch with several *O*-glycosylation sites [29]. Overall, ~15% of the total mass of pro-MMP-9 is derived from glycosylation. Currently, the function of the carbohydrate moieties is unknown.

The paradox of pro-MMP-9 activation

Like all MMPs, pro-MMP-9 is produced in an inactive zymogenic form that requires activation for enzymatic activity. The highly conserved cysteine residue in the propeptide domain (PRCGVPD) in all MMPs is responsible for stabilizing the catalytically inactive pro-enzyme, as this cysteine's thiol coordinates the catalytic zinc ion to maintain latency. Chemical or proteolytic mechanisms can trigger a conformational change that disrupts the Cys-Zn^{2+} interaction [30] allowing the catalytic zinc ion to become available for the catalytic function. Kotra et al. [16] investigated the incremental chemical steps necessary for the 'cysteine switch' event, a process that also requires a conformational change, conceivably within the propeptide domain. Furthermore, the process requires protonation of the side chain of the coordinate cysteine for its departure from the coordination sphere of the metal ion [16].

The activation of pro-MMP-9 has been studied in detail and both proteolytic and non-proteolytic activation mechanisms have been described. A variety of proteases can cleave the propeptide of pro-MMP-9 at specific sites. Stromelysin 1 (MMP-3) is one of the most effective pro-MMP-9 activator, an enzyme that hydrolyzes the Glu^{59}-Met^{60} peptide bond, followed by the Arg^{106}-Phe^{107} amide bond generating the fully active 82-kDa species via an inactive intermediate form of ~85 kDa [31]. Cleavage at the Glu^{59}-Met^{60} site by MMP-3 occurs rapidly [32], consistent with this site being on the surface of the propeptide domain of pro-MMP-9. The second cleavage site is buried and thus requires relaxation of the secondary structure around the activation site, prior to its fitting into the active site of MMP-3 and hydrolysis of the peptide bond [16,33]. It has been reported that the active 82-kDa species is further processed to a ~64-kDa active species by removal of a fragment from the *C*-terminal end [34]. However, the function of this truncated MMP-9 fragment and the precise mechanism involved in its formation remains unknown. Nevertheless, removal of the *C*-terminal region of MMP-9 may alter sensitivity to TIMP-1 inhibition, as this region is the high-affinity binding site for TIMP-1 [27,32].

MMP-2 [35], MMP-7 [36] and MMP-13 (collagenase-3) [37] can also activate pro-MMP-9. MMP-2 and MMP-13 activate pro-MMP-9 by cleaving at the same sites cleaved by MMP-3 [35,37]. Activation of pro-MMP-9 by MMPs is likely to be a cell surface event, because the activating MMPs are known to associate directly or indirectly with the cell surface. For example, pro-MMP-3 is activated by plasmin, which in turn is generated from plasminogen by urokinase plasminogen activator (uPA) bound to the uPA receptor on the plasma membrane. This process can lead to pro-MMP-9 activation via plasmin-activated MMP-3 [38]. With regards to MMP-2 and MMP-13, the activation of pro-MMP-9 by these proteases is also the result of a cascade of zymogen activation initiated on the cell surface by the membrane-tethered MT1-MMP as depicted in Figure 2. Indeed, pro-MMP-2 is activated by MT1-MMP [39] and both MT1-MMP and MMP-2 are able to activate pro-MMP-13 [40]. It is thought that the overall activity of these proteases at the cell surface can accomplish the efficient degradation of interstitial collagen, which is initiated by the collagen-degrading enzymes (MT1-MMP and MMP-13) and is followed by the gelatinase activities of MMP-2 and MMP-9. The co-distribution of these enzymes in many human cancer tissues suggests a role for this proteolytic cascade in degradation of ECM at the pericellular tumor microenvironment.

Several serine proteinases can also activate pro-MMP-9. Recent studies demonstrated that a tissue-associated chymotrypsin-like proteinase is responsible for activation of pro-MMP-9 in skin tissues of patients with chronic unhealed wounds [41]. Pancreatic trypsin-2 isolated from a human carcinoma cell line was shown to be an effective activator of pro-MMP-9 [42]. Both α- and β-chymases derived from mast cells are also pro-MMP-9 activators [43,44]. The activation of pro-MMP-9 by α-chymase was associated with the angiogenesis switch in a mouse model of squamous epithelial carcinogenesis [44].

Although distinct proteolytic pathways can lead to activation of pro-MMP-9, a plethora of studies examining the role of MMP-9 in cellular systems failed to produce evidence of active enzyme (determined as the presence of a ~82-kDa species), even when a particular cellular activity

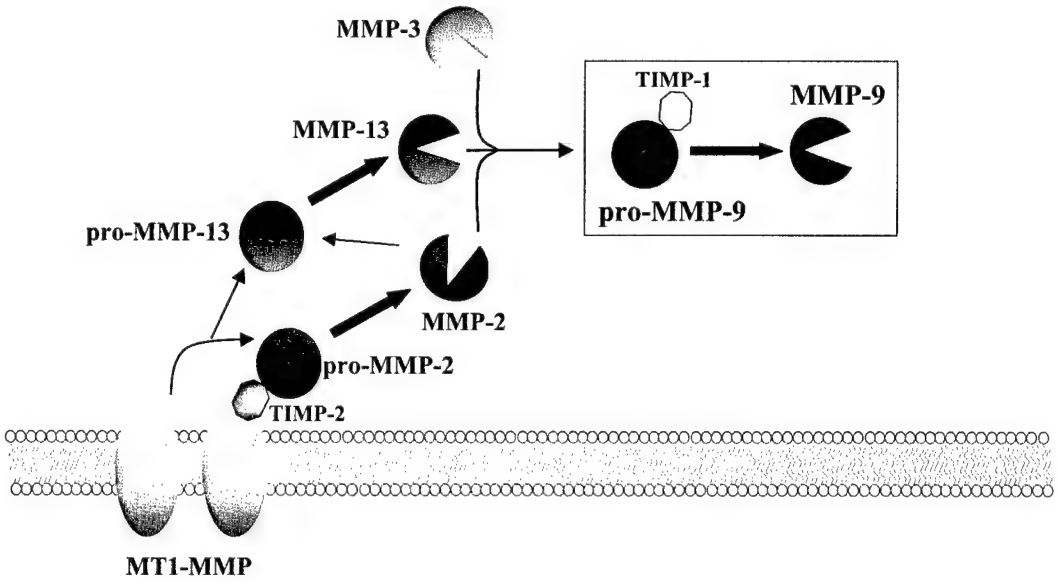

Figure 2. MMP cascade of zymogen activation involved in pro-MMP-9 activation. Pro MMP-9 can be activated by several MMPs including MMP-3 [31,77], MMP-2 [35] and MMP-13 [37]. This cascade of zymogen activation is initiated by MT1-MMP at the cell membrane and requires the action of TIMP-2. It culminates with the generation of MMP-2 and MMP-13, which in turn can activate pro-MMP-9. MMP-3 is another pro-MMP-9 activator, probably the most efficient, but is activated by plasmin via uPAr and uPA on the cell surface (not depicted here). Pro-MMP-9 can be found in a complex with TIMP-1 but the role of this complex on activation is unknown. How pro-MMP-9 can be in proximity to this potential activation cascade is also unknown.

was ascribed to the action of MMP-9. More striking is the fact that even in the absence of detectable active MMP-9, some *in vitro* studies demonstrate an inhibitory effect on cell function related to MMP-9 when its activity is targeted with inhibitory molecules (antibodies or synthetic MMP inhibitors). This lack of correlation between cellular activity and a reliable detection of active MMP-9 represents a major dilemma in our understanding pro-MMP-9 activation and MMP-9 function in the cellular environment.

In cultured cells, is not uncommon to find only pro-MMP-9 even when the same cells are known to contain potential pro-MMP-9 activators. For example, the presence of active MMP-2, a pro-MMP-9 activator [35], does not necessarily correlate with pro-MMP-9 activation even when cells are treated with agents that induce MT1-MMP-dependent pro-MMP-2 activation such as phorbol ester or concanavalin A [45]. Several possibilities may explain this paradox in cultured cells. For instance, TIMPs may inhibit pro-MMP-9 activation by inhibiting the activators [38]. Indeed, some agents that induce pro-MMP-9 gene expression also induce TIMP-1. While MMP-2 is surface-bound, pro-MMP-9 is free in solution and thus inaccessible to potential surface-associated activator. In cultured cells, binding of pro-MMP-9 to the surface is followed by a relatively rapid dissociation (unpublished data from the Fridman lab), a process that would not favor activation by a putative cell surface-associated pro-MMP-9 activator. On the other hand, culture media may dilute soluble activators such as MMP-3. Thus, culture conditions may not favor the interaction between activators and pro-MMP-9. This is in contrast to pro-MMP-2 in which an MT1-MMP/TIMP-2 complex acts as a high-affinity cell surface 'receptor' for pro-MMP-2 allowing for the proximity of MT1-MMP with pro-MMP-2 [46]. It should be noted that a cellular mechanism of pro-MMP-9 activation analogous to that

described for pro-MMP-2 has not yet been described.

In cancer tissues, positive identification of active MMP-9 has also been a challenge. Analyses of tissue extracts derived from malignant tumors by gelatin zymography often reveal high levels of the enzyme, but in many cases the bulk of it exhibits a molecular mass consistent with the latent form. When a lower molecular weight form of MMP-9 is detected, it is unclear whether it represents the truly active form. First, as a glycoprotein, pro-MMP-9 is synthesized as an underglycosylated precursor form that undergoes full glycosylation to the mature species as the enzyme traffics through the secretory pathway [33,47]. Therefore, the precursor form does not exhibit enzymatic activity when tested in activity assays, as it possesses an intact propeptide domain. Second, the activation of pro-MMP-9 by several activators occurs through an intermediate form of ~85 kDa that is generated by cleavage at the Glu^{59}-Met^{60} site, 47 residues upstream of the actual activation site. This species maintains zinc coordination by the cysteine residue within the propeptide and, consequently, is inactive [31]. The presence of these forms in tissue extracts/cell lysates can easily be mistaken for the active species, in particular when using gelatin zymography as the sole method to assess the activation status. Therefore, additional assays should be carried out to determine whether the sample contains truly active MMP-9.

Recent studies have suggested the existence of an alternative mechanism for pro-MMP-9 activation by disengagement of the propeptide domain from the active site without the participation of proteolytic enzymes. This process may occur via oxidative modification of the cysteine side-chain thiol, which would diminish its ability to serve as an effective ligand to the catalytic zinc ion [48,49], or via conformational changes induced by binding to substrate [50]. Either process may result in activation without removal of the inhibitory propeptide domain and consequently generate enzymatic activity in the absence of a noticeable change in molecular mass. However, the efficiency of this activation process and its preponderance in tumor tissues are unknown. Nevertheless, if confirmed, this mechanism may partly explain why the full-length form of MMP-9 (an active enzyme containing a propeptide domain) is usually detected in biological samples.

Pertinent issues regarding assessment of surface-associated pro-MMP-9/MMP-9

Members of the MMP family can be classified into two major groups, secreted and membrane-bound. While the secreted MMPs are released into the extracellular space, the membrane-anchored MMPs are tethered to the plasma membrane. At first glance, the presence or absence of the membrane-anchoring motifs in a particular MMP pinpoints to a differential subcellular localization and consequently to a distinct area of action. Given the significant sequence homology that exists among the members of the MMP family, the inclusion of membrane-anchoring motifs may compensate for their redundancy by targeting relatively similar enzymes to different cellular compartments. From the perspective of enzyme evolution, it is reasonable to assume that the membrane-bound MMPs have evolved to fulfill their proteolytic function at the immediate pericellular space, whereas the secreted MMPs, like MMP-9, have evolved to function in settings that are not directly associated with the cell surface. By having both types of enzymes, the cell would exert a wider range of control over its environment and cellular activities in a more efficient manner. For example, membrane-anchored MMPs, due to their location and high effective concentrations on the plasma membrane, would degrade membrane-associated proteins more efficiently than their secreted counterparts. In contrast, the secreted MMPs would process a subset of substrates not directly associated with the cell surface, thus avoiding unnecessary damage to cellular integrity. Inevitably, the existence of both membrane-anchored and unanchored MMPs is an important achievement for controlling proteolysis at different fronts of the cellular milieu. Thus, surface binding of the secreted MMPs, such as MMP-9, must afford the cells and/or the enzyme with a biological advantage, which does not necessarily imply better substrate environment and/or catalytic activity, as will be discussed later.

From the perspective of spatial localization governed by structural features, it can be proposed that based on the presence of the gelatin-binding domain, gelatinases represent a distinct subgroup among the secreted MMPs. The gelatin-binding domain confers to these enzymes high-affinity binding to extracellular collagen molecules and therefore the secreted gelatinases may bind to the ECM upon release. Under these conditions, the ECM may act as a storage depot for gelatinases released by migratory tumor and/or stromal cells, which can be activated subsequently upon cellular demand. This notion is supported by the fact that the latent forms of the gelatinases bind efficiently to gelatin suggesting that substrate binding is independent of catalytic activity and may precede zymogen activation. If gelatinases are secreted and subsequently bind to the ECM, how do cells regulate their activation and activity at the pericellular space? The studies with the plasminogen activator/plasmin activation cascade, where conversion of plasminogen to plasmin by plasminogen activator involves binding of plasminogen activator to the urokinase plasminogen activator receptor (uPAR) on the cell surface has raised the possibility that a comparable mechanism may also exist for the secreted MMPs. In the case of pro-MMP-2, this issue was resolved by the discovery of a pro-MMP-2 'receptor', formed by the complex of MT1-MMP with TIMP-2, which binds with high affinity to pro-MMP-2 and activates it on the cell surface. Since apparently the gelatin-binding domain of pro-MMP-2 is not involved in the activation process, interactions of pro-MMP-2 via the gelatin-binding domain with the ECM may not influence activation. Pro-MMP-9 cannot bind to TIMP-2 and MT1-MMP cannot bind TIMP-1 and therefore an MT-MMP-dependent mechanism of surface association and activation may not apply to pro-MMP-9.

Another relevant issue concerns the methodology that has been applied to identify surface associated pro-MMP-9/MMP-9. Most studies have used gelatin zymography, a commonly used technique to detect gelatinases in biological samples. Because of the conditions of the assay, gelatin zymography permits detection of both latent and active species and is a technique of high sensitivity (for a detailed description of this technique and its pros and cons the reader is referred to Toth and Fridman [51]). Therefore, special caution should be applied when detecting pro-MMP-9/MMP-9 in plasma membrane preparations and/or in immunoprecipitates by zymography to account for any possibility of sample contamination, in particular after enzyme overexpression. Detection of MMP-9 species with a lower molecular mass in cell lysates may not necessarily represent the true active form as discussed above [47]. This aspect of pro-MMP-9 should be kept in mind when assessing the activation status of secreted or surface-associated MMP-9. Also, specific surface binding of MMP-9, and of any other MMP under study, would be better supported by additional ligand binding studies to determine the true affinity and kinetics of association, when possible.

Mechanisms of MMP-9 surface association

Surface associated MMP-9 has been identified in a variety of human and murine normal and tumor cells, including neutrophils [52], endothelial [53–56], keratinocytes [57], breast epithelial [47,53], breast cancer [58], pancreatic cancer [59], ovarian cancer [60], prostate cancer [61], fibrosarcoma [62,63] and mouse mammary carcinoma [64] cells. Earlier studies with non-malignant and malignant cell lines demonstrated that factors that induce pro-MMP-9 gene expression result in a fraction of the secreted enzyme to be associated with the cell surface. Breast epithelial MCF10A cells were shown to contain pro-MMP-9 on the cell surface after treatment with phorbol ester [47]. Although phorbol ester also stimulates expression and secretion of TIMP-1, the plasma membrane fraction of MCF10A cells contained only pro-MMP-9, whereas the pro-MMP-9/TIMP-1 complex was detected in the culture media suggesting the possibility that surface association of pro-MMP-9 can somehow preclude TIMP-1 from binding to the zymogen. Although pro-MMP-9 was consistently found on the cell surface, active MMP-9 forms could not be detected. However, this study [47] reported the surface association of a low molecular weight form of MMP-9, with a molecular mass similar to that exhibited by the active species ($\sim$82 kDa). Further analyses

revealed that this species is a precursor of the underglycosylated form of the zymogen that could also bind to the plasma membrane, albeit this form was not secreted [47]. The significance of this finding has not been established, but may be a consequence of enzyme overexpression. Nevertheless, this study revealed a potential pitfall when analyzing surface-associated MMP-9 and raised the importance of determining the true nature of the enzyme species detected on the cell surface, in particular under condition that induces enzyme expression. Studies with the human MCF-7 breast cancer cell line demonstrated the presence of surface-associated MMP-9 [58]. Expression of MMP-9 was induced by insulin-like growth factor I (IGF-I) and correlated with enhanced cell migration toward IGF-I. Interestingly, whereas pro-MMP-9 was detected in the culture supernatant, on the cell surface a non-specific MMP activity assay, based on a fluorogenic peptide substrate, indicated enzymatic activity. However, the actual active MMP-9 form was not identified. Ovarian cancer OVCA 429 cells exposed to epidermal growth factor (EGF) showed a significant association of pro-MMP-9 with the cell surface [60]. In these cells, surface association of pro-MMP-9 was independent of the level of enzyme expression, but was dependent on EGF treatment suggesting that the binding component was induced by the growth factor. Studies in prostate carcinoma PC3 cells showed that exposure to transforming growth factor-β1 (TGF-β1) resulted in enhanced secretion and activation of pro-MMP-9 [61]. A fraction of both the latent and active MMP-9 was detected on the cell surface. Although the mechanism leading to pro-MMP-9 activation in the presence of TGF-β1 was not addressed, TGF-β1 also promoted membrane-associated plasmin activity consistent with a possible cascade of zymogen activation on the cell surface leading to activation of the surface bound pro-MMP-9 by a mechanism involving plasmin. It should be noted that plasmin is not a direct activator of pro-MMP-9 and only produces a single cleavage in the propeptide domain generating the inactive intermediate form, sometimes mistaken for the fully active species [38]. Collectively, these studies confirmed the notion that MMP-9 can associate with the cell surface in cultured cells and stimulated the search for MMP-9-binding proteins.

Binding of MMP-9 to the pericellular extracellular matrix

Pro-MMP-9 contains a gelatin-binding domain that is known to mediate binding of the enzyme to denatured collagen. This property is widely used for isolation of pro-MMP-9 from biological samples by means of a gelatin-affinity chromatographic procedure. As discussed above, the existence of this domain must permit the *in vivo* interaction of MMP-9 with collagen molecules of the ECM. Olson et al. [53] showed that the binding of pro-MMP-9 to the cell surface in various cell lines was mediated via a surface-associated α2(IV) chain of collagen IV, a subunit of the basement membrane collagen IV molecule. These findings were consistent with immunohistochemical studies showing the presence of MMP-9 on the surface of cancer cells in tissues of breast carcinomas [65] and in the basement membrane of skin tumors [44,66]. Also, in migrating cultured human bronchial epithelial cells (HBEC), both pro-MMP-9 and MMP-9 were detected in the insoluble ECM at the leading edge in close association with collagen IV [67]. In addition, α2(IV) molecules can be detected on the surface of cultured breast epithelial cells [68].

Binding of pro-MMP-9 to α2(IV) is high-affinity ($K_d \sim 22$ nM) and complexation with TIMP-1 does not affect binding, indicating that the hemopexin-like domain is not critical for the association. Thus, the gelatin-binding domain must be an essential requirement for this interaction. However, pro-MMP-2, which also possesses a gelatin-binding domain shows a significantly lower affinity for the α2(IV) chain, compared to pro-MMP-9 [53]. Possibly, other sites and/or the overall enzyme conformation may also regulate the interactions with the α2(IV) chain. Recently, the hemopexin-like domain of pro-MMP-9 was shown to participate in binding of the enzyme to gelatin [69]. Since both gelatinases can cleave denatured collagen IV with similar efficiencies, association of pro-MMP-9 with α2(IV) may serve to localize pro-MMP-9 at the pericellular space. Since the existence of monomeric α2(IV) chains in the extracellular space remains an unresolved issue [68], it is conceivable that binding of pro-MMP-9 to the α2(IV) chain is mediated by sites that are cryptic in native collagen IV, which are only

exposed after partial unwinding of the collagen IV molecule.

Functionally, binding of pro-MMP-9 to the α2(IV) chain does not appear to play a direct role in regulation of zymogen activation and/or enzyme inhibition [53]. It is also unknown whether activity of MMP-9 is altered in any way by this interaction. Recently, binding of pro-MMP-9 to gelatin was shown to induce pro-MMP-9 activation via a non-proteolytic mechanism, resulting in the acquisition of catalytic activity without removal of the propeptide domain [50]. Whether binding to the α2(IV) chain can also produce a similar effect remains to be determined. Nevertheless, the high-affinity binding of pro-MMP-9 to α2(IV) may serve to favor the targeting of the zymogen to the pericellular ECM.

Binding of MMP-9 to the hyaluronan receptor CD44

The active form of MMP-9 has been shown to associate with the cell surface via the hyaluronan receptor CD44 in cultured murine mammary carcinoma and human melanoma cells [64,70]. CD44 is a heavily glycosylated transmembrane protein that as a consequence of extensive alternative splicing exists in multiple variant forms. CD44 has been implicated in tumor growth, invasion and metastasis [71] and thus, association of MMP-9 with CD44 has been suggested to link cell adhesion with pericellular proteolysis [64]. Indeed, association of MMP-9 with CD44 has been shown to promote tumor cell invasion *in vitro* and in experimental metastasis assays [64]. Furthermore, expression of a soluble CD44 receptor abrogated invasion *in vitro* and *in vivo*, and also inhibition of MMP-9 activity by an inhibitory antibody or antisense obliterated the invasive ability of the cells expressing CD44 and surface-bound MMP-9 [64]. MMP-9 bound to the CD44 receptor was also shown to process latent TGF-β to the biologically active form [8]. Generation of active TGF-β on the cell surface may enhance tumor growth and metastasis by promoting a degradative phenotype [72] and by inducing angiogenesis [8].

Association of MMP-9 with CD44 was demonstrated by co-immunoprecipitation experiments and co-localization of the proteins on the cell surface by immunofluorescence [64,70], but the relative binding affinity and specificity of the MMP-9/CD44 interaction remain unknown. Interestingly, MMP-9 bound to CD44 was reported as being in the active form, as determined by gelatin zymography [64], but the mechanism of activation and whether it precedes binding to CD44 is unknown. A recent study reported the interaction of MT1-MMP with CD44 [78]. We can speculate that association of CD44 with pro-MMP-9 may bring together this zymogen close to the MT1-MMP/MMP-2 axis on the cell surface and consequently promote pro-MMP-9 activation as depicted in Figure 2.

Binding of MMP-9 to RECK and LRP

Recent evidence indicates that binding of MMP-9 to the cell surface may also negatively regulate enzyme function. Thus, the commonly held view that surface association of proteases is advantageous for pericellular proteolysis may not be true under all circumstances. In fact, recent studies show that two distinct membrane-tethered proteins may negatively influence MMP-9 activity in the pericellular microenvironment. These membrane proteins are RECK (reversion-inducing-cysteine-rich protein with Kazal motifs) [79] and LRP (low-density lipoprotein receptor-related protein) [73]. The *RECK* gene encodes for a glycosylphosphatidylinositol (GPI)-anchored cysteine-rich glycoprotein containing serine protease inhibitor-like domains and regions with weak homology to epidermal growth factor (EGF) [74]. RECK expression is down regulated in a variety of tumor cell lines and in cells transformed by a variety of oncogenes, suggesting a tumor suppressing function for RECK. LRP is a member of the LDL receptor family known to mediate endocytotic intake of extracellular proteins as diverse as lipoproteins, protease-inhibitor complexes, proteases, growth factors, ECM components, viruses, and bacterial toxins. More than 30 ligands were found to bind LRP with high affinity, indicating the broad role that LRP plays in the regulation of protein function by internalization.

Expression of RECK in various cell lines inhibited *in vitro* invasion and metastasis *in vivo* without significant effects on the proliferative capacity of the cells. Subsequently, it was found

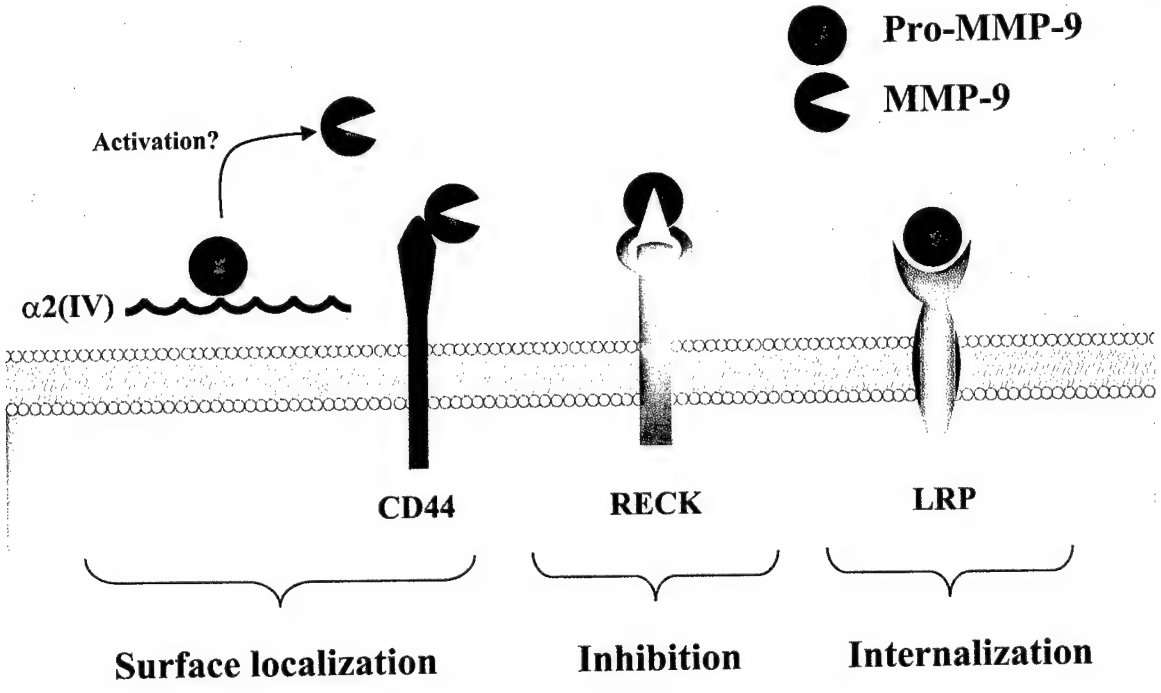

Figure 3. Reported proteins involved in pro-MMP-9/MMP-9 cell surface association. These diverse proteins play distinct roles in MMP-9 function including surface localization, inhibition and internalization. In the case of α2(IV) and CD44, it is unknown whether the interaction is associated with zymogen activation or inhibition. However, CD44-bound MMP-9 was shown to be in the active state.

that expression of RECK was associated with specific reduction in the amounts of secreted pro-MMP-9, while no differences were observed in pro-MMP-2 secretion. Although RECK has no homology to TIMPs, a soluble RECK protein was shown to inhibit the enzymatic activity of MMP-9 [74], MMP-2 and MT1-MMP [75], albeit with a lower affinity than TIMPs. The mechanism by which RECK inhibits MMP activity is unclear, given its homology to serine protease inhibitors, but the inhibitory effects are not specific since various MMPs, both soluble and membrane-anchored, are equally inhibited [75]. In contrast, there appears to be specificity in recognition of pro-MMP-9 by a soluble recombinant RECK [74]. Whether membrane-anchored RECK can bind pro-MMP-9 on the cell surface and regulate its activation remains unclear. The inhibitory effect of RECK on MMP function is believed to be the cause of some of its tumor suppressing effects and also for the lethal consequence of *RECK* gene ablation in knockout mice [75]. The *RECK* null mice fail to develop beyond the embryonic stage and exhibit severe malformation of mesenchymal and vascular tissues, which have been ascribed to reduced MMP activity when compared to the wild type *RECK* embryos. Proteins like RECK have been proposed to represent a new class of protease inhibitors that act specifically on the cell surface to control enzymatic activity in the pericellular space, as opposed to soluble MMP inhibitors, which are secreted [75]. Thus, surface-bound molecules that can act as protease inhibitors may add an extra level of regulation during pericellular proteolysis by trapping and inhibiting secreted MMPs on the cell surface.

LRP is responsible for the internalization of a variety of ligands including pro-MMP-9, which was shown to bind with high affinity to purified LRP either as a free enzyme or in complex with TIMP-1 [76]. Binding of pro-MMP-9 to LRP is inhibited by RAP (receptor associated protein), a protein that inhibits ligand binding to LRP [73]. Upon binding to embryonic fibroblasts expressing

LRP, the MMP-9/TIMP-1 complex is internalized as a function of time, a process that is followed by degradation of the complex in a chloroquine-dependent mechanism [76]. These studies suggest a role for LRP in control of the level of MMP-9 in the pericellular environment by promoting clearance and subsequent catabolism of the latent enzyme and the enzyme/inhibitor complex. There is not yet evidence that LRP can promote internalization of the active enzyme or the active enzyme in complex with TIMPs.

Concluding remarks

MMP-9 is one of the major MMPs involved in tumor progression and compelling evidence highlight MMP-9 as a major regulator of tumor angiogenesis. Therefore, investigations of the mechanisms regulating its function in cancer have raised considerable interest. However, in contrast to its homologue pro-MMP-2, elucidation of the processes that regulate pro-MMP-9 function in the extracellular space remains a scientific challenge. Structurally, pro-MMP-9 possesses unique features and their roles in enzyme function have not yet been completely elucidated. Although activation of pro-MMP-9 is accomplished by a variety of proteolytic enzymes, the relevant mechanism(s) of pro-MMP-9 activation in tumor tissues and in particular at the cell surface remains elusive. Most puzzling of all is the consistent inability to detect active MMP-9 in cultured cells and tissues. The association of pro-MMP-9 with the cell surface has been an area of intense research and several proteins have been identified as potential mediators of MMP-9 cell surface binding. These proteins mediate surface targeting (α2(IV), CD44), inhibition (RECK) and internalization (LRP) of pro-MMP-9/MMP-9 and thus, regulate distinct aspects of MMP function. This demonstrates the complex regulation of pro-MMP-9 at the pericellular space. Figure 3 summarizes the variety of mechanisms that may be involved in the association of pro-MMP-9 and MMP-9 to the cell surface and their effect on enzyme function. Understanding how both tumor and stromal cells sort out among these mechanisms and how they utilize them to most effectively regulate MMP-9 activity at the pericellular space represent the next big challenge in MMP-9 research.

Key unanswered questions

1. Pro-MMP-9 can be activated by several MMPs that are known to be surface associated (Figure 2). What are the mechanisms that bring pro-MMP-9 in close association to this cascade of zymogen activation?
2. Is there a cell surface mechanism of pro-MMP-9 activation analogous to that described for pro-MMP-2? Are there MT-MMPs involved in pro-MMP-9 activation?
3. Pro-MMP-9 forms a complex with TIMP-1. What is the role of this complex in surface association and/or activation?
4. Are there other types of surface-bound proteases (serine, etc.) that can activate pro-MMP-9?
5. What are the structural requirements for pro-MMP-9 surface association?
6. Pro-MMP-9 is known to form disulfide-bonded homodimers [33]. What is their role in surface association and activation?

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